# The role of Wnt signalling in actin dynamics during synapse formation

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# Abstract

Wnt secreted proteins play key roles in the formation of neuronal circuits by regulating axon guidance, axonal remodelling, dendritogenesis and synaptogenesis. We have previously shown that Wnts promote axonal remodelling through the regulation of actin cytoskeleton and the formation of dendritic spines. However, the downstream events modulated by Wnts to regulate actin dynamics during these processes remain elusive.

Here, we identified the actin-capping protein Epidermal growth factor receptor kinase substrate 8 (Eps8) as a direct interactor of Dishevelled-1 (Dvl1), a key scaffold protein and integrator of Wnt signalling. Expression of Eps8 mimics Dvl1-induced axonal remodelling by promoting enlargement and F-actin accumulation in growth cones from dorsal root ganglia (DRG) neurons. Importantly, we show that Eps8 is required for Wnt3a-mediated axonal remodelling. Our findings demonstrate that Eps8 is a downstream effector of Wnt signalling during axonal remodelling.

Dendritic spine morphogenesis critically depends on actin dynamics, a process that is modulated by signalling molecules and neuronal activity through poorly described mechanisms. Here we report that Eps8 is required for spine morphogenesis in hippocampal neurons. Our gain- and loss-of-function studies demonstrate that Eps8 promotes the formation of dendritic spines but inhibits filopodium formation. However, Eps8 does not affect spine growth nor modulates Dvl1-mediated spine enlargement, indicating that Eps8 regulates spine formation through a Wnt-independent pathway. Loss of function of Eps8 results in increased actin polymerization, but also actin turnover within dendritic spines, as revealed by free-barbed end and FRAP assays, consistent with a role for Eps8 as an actin-capping protein. Interestingly, Eps8 promotes the localisation of excitatory synapses on spines, without affecting the total number of synapses or basal synaptic transmission. Importantly, Eps8 silencing impairs the structural and functional plasticity of synapses induced by long-term potentiation. These results demonstrate a novel role for Eps8 in spine formation and in activity-mediated synaptic plasticity.

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# Acknowledgements

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# Declaration of authorship

I, Eleanna Stamatakou, confirm that all data presented in this thesis are my own with the exception of the electrophysiological recordings, which were performed in collaboration with Prof. Alasdair Gibb and Dr. Aude Marzo. Prof. Gibb and Dr. Marzo did all the whole-cell patching, whereas I prepared the cultures and the solutions needed, performed the treatments and carried out the analyses.

Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

Part of the introduction has been published in the form of a review (Stamatakou & Salinas 2014) and some of the data presented in Chapter 5 and 6 has been published in a manuscript, where I am the first author (Stamatakou et al 2013). Recently, we also published a manuscript based on the results included in Chapters 3 and 4, where I am sharing the first authorship with Dr. Monica Hoyos-Flight.

## Publications

**Stamatakou E**, Hoyos-Flight M, Salinas PC (2015). Wnt3a regulates actin dynamics during axon remodelling through the actin-binding protein Eps8. PLoS One. 7;10(8):e0134976.

**Stamatakou E** and Salinas PC (2014). Postsynaptic assembly: A role for Wnt signaling. Dev Neurobiol. 74(8):818-27.

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# Abbreviations

**Abi1: Abelson interactor 1**

**ABP: Actin-binding protein**

**AD: Activator domain of GAL4**

**AMPA: α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid**

**APC: Adenomatous polyposis coli**

**Arp2/3: Actin-related protein 2/3**

**AZ: Active zone**

**BD: DNA-binding domain of GAL4**

**BDNF: Brain-derived neurotrophic factor**

**CaMK: $Ca^{2+}$/calmodulin-dependent protein kinase**

**CGNs: Cerebellar granule neurons**

**cLTP: Chemical Long Term potentiation**

**CNS: Central nervous system**

**CP: Capping protein**

**CRD: Cysteine-rich domain**

**CYFIP1: cytoplasmic FMR1 interacting protein 1**

**CytoD: Cytochalasin D**

**DISC1: Disrupted-in-Schizophrenia 1**

**DRG: Dorsal root ganglion**

**Dvl1: Dishevelled 1**

**EGFP: Enhanced green fluorescent protein**

**Epac2: Exchange protein activated by cAMP**

**Eps8: Epidermal growth factor receptor kinase substrate 8**

**Eps8TM: Eps8 carrying a triple mutation**

**ER: Endoplasmic reticulum**

**F-actin: Filamentous actin**

**FGF: Fibroblast growth factor**

**FRAP: Fluorescence recovery after photobleaching**

**FRET: Förster resonance energy transfer**

**Fz: Frizzled receptor**

**G-actin: Globular actin**

**GABA: γ-aminobutyric acid**

**GAP: GTPase activating protein**

**GC: Growth cone**

**GEF: guanine-nucleotide exchange factor**

**Gsk3: Glycogen synthase kinase 3**

**HFS: High frequency stimulation**

**IF: Immuno-fluorescence**

**IPSC: Inhibitory postsynaptic current**

**IRSp53: Insulin receptor tyrosine kinase substrate p53**

**KD: Knock down**

**KO: Knock out**

**LatB: Latrucullin B**

**LEF: Lymphoid Enhancer Factors**

**LFS: Low frequency stimulation**

**LIMK1: LIM domain kinase 1**

**LRP: Low-density lipoprotein receptor-related protein**

**LTD: Long-term depression**

**LTP: Long-term potentiation**

**mEPSC: Mini excitatory postsynaptic currents**

**mGluR: Metabotropic glutamate receptor**

**NL: Neuroligin**

**NMDA: N-methyl-D-aspartate receptor**

**NMJ: Neuromuscular junction**

**NRX: Neurexin**

**NT-3: Neurotrophin-3**

**PKC: Protein kinase C**

**PSD: Postsynaptic density**

**PSD95: Postsynaptic density protein 95**

**Psmd8: 26S proteasome non-ATPase regulatory subunit 8**

**Rap: Ras-related protein**

**SALM: Synaptic adhesion-like molecule**

**Sfrp: Secreted frizzled-related protein**

**sGluA1: Surface glutamate receptor subunit GluA1**

**SH3: SRC Homology 3 Domain**

**shRNA: short-hairpin RNA**

**SIRP: Signal regulatory protein**

**SMF: Synaptosomal membrane fraction**

**SV: Synaptic vesicle**

**SynCAM: Synaptic cell adhesion molecule**

**TCF: T-cell factors**

**TrkB: Neurotrophic tyrosine kinase receptor B**

**VASP: Vasodilator-stimulated phosphoprotein**

**VGCC: Voltage-gated calcium channels**

**vGlut: Vesicular glutamate transporter**

**WCR: WAVE regulatory complex**

**Y2H: Yeast two-hybrid**

# Chapter 1:

# General Introduction

# 1.1 Introduction

The formation of neuronal networks is a complex process that requires the precise and coordinated behaviour of neurons as a result of the activation of several signalling pathways. Neurons extend their axons in search for their synaptic targets. Once axons have reached their synaptic partners, they begin to assemble synaptic contacts by the recruitment of hundreds of pre- and postsynaptic components (McAllister 2007, Sheng & Kim 2011). The interplay between secreted factors, adhesion molecules and intracellular signalling molecules determine the formation of synapses in which assembled presynaptic terminal are in perfect apposition to the postsynaptic side.

Chemical synapses are the functional units within a neuronal circuit. These highly specialized cellular junctions are the sites of electric communication that pass information directly from the presynaptic axonal terminals to postsynaptic regions (eg. another neuron, a muscle or a gland) through the conversion of an electrical input to a molecular signal and then back to an electiral signal on the postsynaptic neuron. The arrival of the action potential to the presynaptic bouton triggers the release of chemical neurotransmitters into the synaptic cleft. Binding of the neurotransmitter by specific receptors localized on the postsynaptic membrane leads to the depolarisation of the postsynaptic neuron and downstream signalling changes.

Synapses are dynamic structures that form, undergo morphological changes and disassemble during development. It is widely accepted that new information in the brain is stored by the strengthening or weakening of existing synapses, which can be accompanied by the formation and/or the elimination of synapses. This structural plasticity is believed to be the basis of information storage in the brain (Bosch & Hayashi 2011, Kasai et al 2010, Segal 2010). Thus, studying the molecular mechanisms that control synapse development, maturation and plasticity is fundamental for understanding brain functions, including learning and memory.

Actin cytoskeleton plays a critical role in structural plasticity in the developing brain. The actin cytoskeleton is essential for maintaining cell shape. In addition to its structural role, the dynamic properties of actin cytoskeleton modulate a variety of processes within the cell from cell division and migration to protein

trafficking. In neurons, actin cytoskeleton has key roles during neuritogenesis and neurite branching. In addition, actin plays a role in synapse formation, growth and elimination. In mature neurons, actin is the most abundant cytoskeletal protein at synapses and its regulation is important for synapse function and plasticity.

This thesis examines the role of Wnt signalling in actin dynamics during synapse formation. Wnt proteins are well-known secreted signalling molecules that regulate axonal and dendritic outgrowth, as well as synapse formation (Budnik & Salinas 2011, Park & Shen 2012, Rosso & Inestrosa 2013). During the last decade great progress has been made in discovering the signalling pathways that regulate these processes. However, we have little understanding of how Wnts modulate the actin cytoskeleton to regulate axonal remodelling and synaptic assembly. To identify new molecules involved in Wnt-mediated axonal remodelling and postsynaptic assembly, we performed a yeast two-hybrid screen, using Dishevelled 1 (Dvl1) protein as bait – a scaffold protein central to all Wnt pathways identified so far. Several candidate interactors were identified. We focused on the Epidermal growth factor receptor kinase substrate 8 (Eps8), a protein that plays multiple roles in the regulation of actin dynamics. The findings presented in my thesis demonstrate that Eps8 acts downstream of Dvl1 to regulate actin dynamics to regulate Wnt-mediated axonal remodelling. In contrast, Eps8 is required postsynaptically for proper formation of dendritic spines possibly independently of Dvl1 and therefore Wnt signalling.

In this introduction chapter I will review the current knowledge on the role of actin-binding proteins during growth cone steering, synapse formation and synaptic plasticity. In addition, the function of Wnt signalling in synapse formation will be also reviewed in detail.

## 1.2 Synapse structure and function

Glutamatergic synapses are tiny (<1μm) highly asymmetric specialised junctions composed of a presynaptic terminal and a postsynaptic side and represent the majority of excitatory contacts in the brain. The presynaptic terminal contains synaptic vesicles (SVs), which are filled with the neurotransmitter glutamate. When a presynaptic neuron fires an action potential, SVs fuse with the plasma

membrane and glutamate is released into the synaptic cleft. Once released, glutamate binds to postsynaptic glutamate receptors, which contain intracellular domains embedded in the postsynaptic density (PSD). Glutamate receptor activation, mainly N-methyl-D-aspartate (NMDA) and α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors, leads to ion influx, local depolarization and activation of postsynaptic signalling cascades.

A typical presynaptic terminal is characterized by an active zone (AZ), a dense region located behind the plasma membrane that directly faces the synaptic cleft, and an associated cluster of vesicles embedded in an actin-rich area (Figure 1.1A and 1.1D). Vesicle fusion and neurotransmitter release occurs within the active zone. Few synaptic vesicles are adjacent to the active zone and are referred to as docked vesicles. Only a subset of docked vesicles are released upon the arrival of an action potential called the readily releasable pool (RRP) of vesicles. Two additional pools exist in the presynaptic terminal: the recycling pool – which contains vesicles resealed in moderate stimulation - and the reserve pool, which is the larger one and contains vesicles released only upon very strong stimulation (Rizzoli & Betz 2005). Thus, the organisation of the presynaptic terminal ensures that release of neurotransmitter occurs in close proximity to their postsynaptic receptors.

Spines are small actin-rich protrusions present along the dendrite, which have a neck and a head, that receive excitatory input. They come in a wide range of sizes and shapes, with lengths varying from 0.3 to 2 μm and volumes from 0.001 to 1 $\mu m^3$ (Arellano et al 2007, Benavides-Piccione et al 2013, Harris & Stevens 1988, Lambert et al 1989). It has become very evident that spine size and synaptic function are closely associated, since large spines contain a high content of AMPA receptors (AMPARs), the main glutamate receptors that mediate fast synaptic transmission in the CNS, whereas small spines have fewer AMPARs (Matsuzaki et al 2001). Dendritic spines are widely categorized into 3 groups based on their morphology: thin, filopodium-like protrusions (“thin spines”), short spines without a defined spine neck (“stubby spines”) and spines with a large bulbous head (“mushroom spines”) (Figure 1.1B) (Bourne & Harris 2008). It is widely accepted that the size and the shape of spines reflects differences in spine maturity and synaptic strength. In particular, small spines with a filopodium-like morphology are considered to be more plastic and are

those that respond to neuronal activity (Bourne & Harris 2007). In contrast, large mushroom-shaped spines usually have a bigger PSD with higher density of AMPARs (Bourne & Harris 2008, Matsuzaki et al 2001), indicating that they bare stronger synapses.

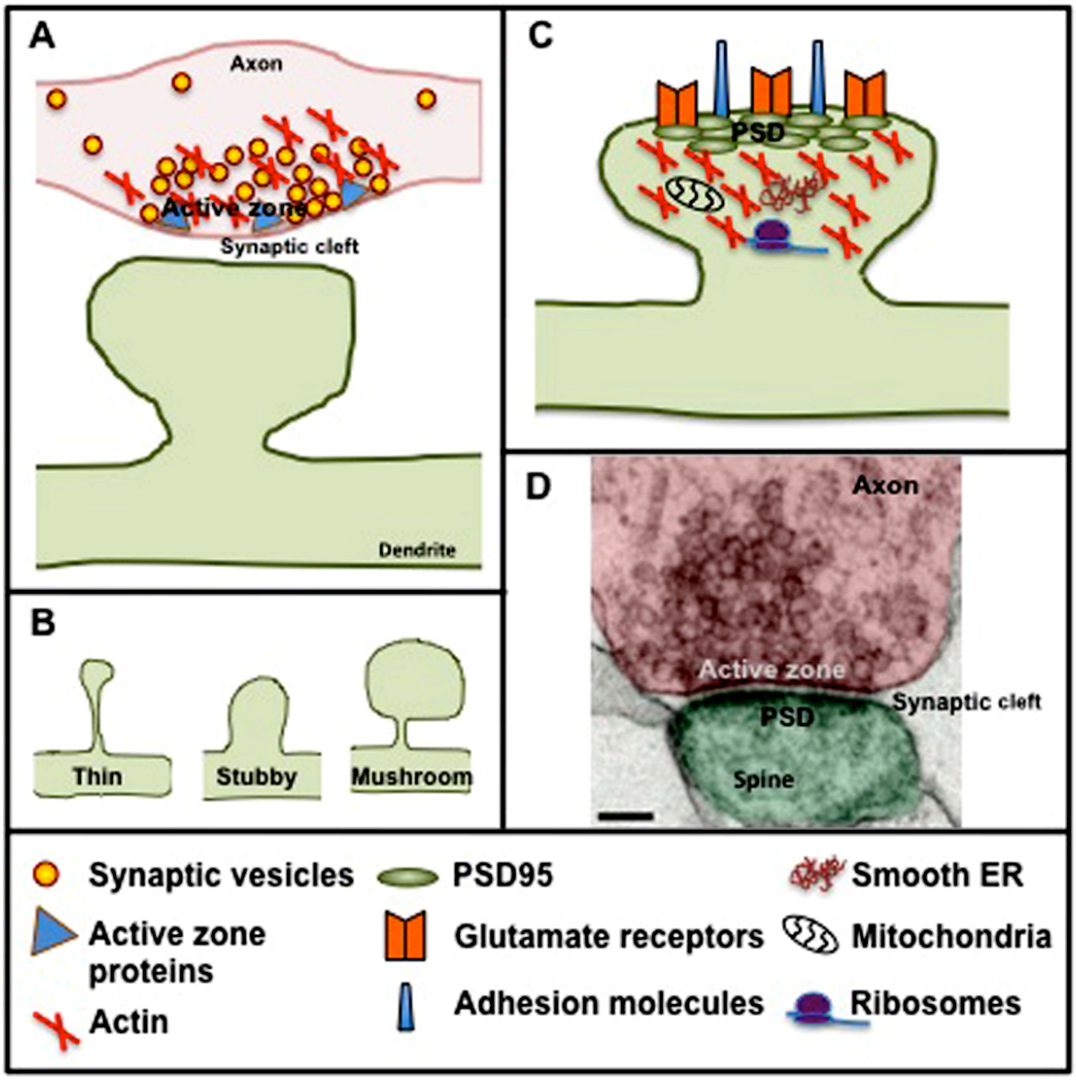



**Figure 1.1: The structure of an excitatory synapse.** Glutamatergic synapses are composed of a pre- and a postsynaptic terminal. (A) The presynaptic sites contains active zone (AZ) proteins and SVs embedded in an actin-rich area. (B) Dendritic spines are categorized into: thin spines (filopodia-like protrusions), stubby spines (short spines without a neck) and mushroom spines (large spines with a bulbous head). Spine heads contain the postsynaptic density (PSD), where receptors, adhesion molecules, and ion channels are anchored (C). In spine heads also are found several organelles, such as smooth endoplasmic reticulum, mitochondria and polyribosomes. (D) EM picture depicting a presynaptic bouton apposed to a dendritic spine. Image from Ellen Dickins PhD thesis. Scale bar: 0.1 μm.

Spine heads contain all the postsynaptic machinery, including glutamate receptors, the postsynaptic density (PSD), a wide variety of organelles, such as smooth endoplasmic reticulum, mitochondria and polyribosomes and an array of

signalling molecules (Han & Kim 2008, Sala & Segal 2014, Sheng & Hoogenraad 2007, Sheng & Kim 2011, Yasuda 2012). The PSD is often found at the tip of the dendritic spine head in direct apposition to the presynaptic terminal and functions as a postsynaptic organizer where receptors, adhesion molecules, and ion channels are anchored (Figure 1.1C and 1.1D)(Kennedy et al 2005, Sheng & Hoogenraad 2007). Spine necks (~0.2 μm wide) are considered to act as a diffusion barrier for synaptic molecules and ions (Araya et al 2006, Grunditz et al 2008, Tonnesen et al 2014). This organisation and morphology of dendritic spines provides neurons with an autonomous postsynaptic compartment where restricted local synaptic signalling could take place. This compartmentalization protects the cell from high $Ca^{2+}$, which enters into the spine upon synaptic activation (Grunditz et al 2008, Korkotian & Segal 2007, Segal 2010). Importantly, the presence of the postsynapse within a dendritic spine allows each synapse to function independently from one another.

## 1.3 Assembly and maturation of glutamatergic synapses

Recent studies have shown that presynaptic and postsynaptic sides are composed of a huge number of proteins (Husi et al 2000, Morciano et al 2009, Satoh et al 2002, Walikonis et al 2000, Weingarten et al 2014). For instance, mass spectroscopy experiments revealed that the PSD only contains around 600 different proteins (Sheng & Hoogenraad 2007, Sheng & Kim 2011). Presynaptically, a variety of proteins are required for SVs recruitment, fusion and recycling (Morciano et al 2009, Sudhof 2012, Weingarten et al 2014), whereas postsynaptically, glutamate receptors and other ion channels are anchored to the PSD via a large number of scaffolding proteins (Husi et al 2000, Satoh et al 2002, Walikonis et al 2000). Thus, synapse formation requires the coordinated recruitment of hundreds of proteins and the assembly of multi-molecular complexes important for synaptic signalling. However, although synaptogenesis is a critical step in neuronal circuit function, the molecular mechanisms that govern synapse formation and maturation are partially understood.

### 1.3.1 Axo-dendritic contact & recruitment of synaptic proteins

For the formation of a synapse an axo-dendritic contact is required. A number of possible types of contacts have been described, including those initiated by axonal and dendritic filopodia (Gallo 2013), which are highly motile protrusions that represent neuronal antennas that probe the local environment in search for synaptic targets. Contacts made from filopodia are highly unstable and transient, but a small subset of these contacts become stabilized and new synapses emerge.

Initial studies have reported that presynaptic assembly occurs prior to postsynaptic development. In particular, upon axon-dendritic contact presynaptic components, including the glutamate transporter vGlut1 and vesicular proteins such as Vamp2 and Synapsin1, are recruited to the site of contact within 10 mins (Friedman et al 2000, McAllister 2007). The postsynaptic scaffold protein PSD95 is recruited to synaptic vesicle recycling sites after about 30 mins, an event that is followed by the accumulation of NMDARs and AMPARs (Friedman et al 2000). However, according to another study, pre- and postsynaptic components are recruited simultaneously at the site of contact within 10 mins (Washbourne et al 2002). A more recent study, in contrast, suggested that accumulation of postsynaptic proteins precede the recruitment of presynaptic proteins (Gerrow et al 2006). Together, these findings suggest that multiple mechanisms exist for the recruitment of pre- and postsynaptic proteins to future synaptic sites.

### 1.3.2 Synapse Maturation: focus on the postsynaptic side

Although synapses can be rapidly assembled, synapse maturation is a prolonged process that requires the recruitment of several synaptic components that are involved in synaptic strengthening. The most profound event in the maturation of glutamatergic synapses is the change in their localisation along the dendrite and the incorporation of AMPARs (and other receptors?). Synapses are initially assembled on dendritic filopodia and dendritic shafts, but later on are primarily located on dendritic spines. In addition, when synapses are just formed, they mainly contain NMDARs but not AMPARs (silent synapses). The subsequent recruitment of AMPARs to the postsynaptic membrane augments synaptic transmission (Kerchner & Nicoll 2008). Thus, dendritic spine morphogenesis and

AMPARs delivery to synapses is a critical event for glutamatergic synapse maturation (Gerrow & El-Husseini 2006, McMahon & Diaz 2011, Tada & Sheng 2006).

Three different models have been proposed for the formation of dendritic spines (Figure 1.2) (Ethell & Pasquale 2005, Yuste & Bonhoeffer 2004). The most accepted model suggests that dendritic spines originate from dendritic filopodium maturation upon axonal contact (Marrs et al 2001, Vaughn & Smyth 1989, Ziv & Smith 1996). The second model involves the appearence of spines from excitatory synapses located on the dendritic shaft (Harris 1999, Miller & Peters 1981). The third model proposes that dendritic spines can also form even without synaptic contact (Knott et al 2006, Sotelo 1990).

### Filopodial model

During early development neurons form many filopodia that are highly motile (Dailey & Smith 1996, Dunaevsky & Mason 2003, Lendvai et al 2000, Ziv & Smith 1996). As synaptogenesis proceeds, the number of filopodia declines with a concomitant increase in the number of dendritic spines, suggesting that filopodia are the precursors of dendritic spines. Indeed, time-lapse recordings demonstrated that filopodia upon axonal contact are transformed into spines (Dailey & Smith 1996, Maletic-Savatic et al 1999, Marrs et al 2001, Okabe et al 2001, Trachtenberg et al 2002, Ziv & Smith 1996). According to this model, dendritic filopodia represent the environmental sensors of dendrites, searching for an appropriate contact with their future presynaptic partner.

### Spine formation from the dendritic shaft (Miller-Peters model)

Spines – in particular stubby spines – can also arise from shaft synapses (Miller & Peters 1981). This model of spinogenesis stems from the observation that during early development the majority of postsynaptic sides are located on short dendritic protrusions (stubby spines) that later acquire a mushroom shape (Miller & Peters 1981). Additional studies demonstrated that in young neurons most synapses are located on the dendritic shaft rather than on filopodia (Boyer et al 1998, Fiala et al 1998, Harris 1999, Reilly et al 2011). As neurons mature, the number of synapses located on spines increases with a concomitant decrease in the number of shaft synapses. According to this model, axonal filopodia may be

involved in probing the local environment for appropriate contacts on the dendritic shaft. Indeed, it has been shown that axonal filopodia form contacts with the dendritic shaft (Fiala et al 1998). Importantly, this model has been supported by the finding that spines form from shaft synapses (Dailey & Smith 1996, Marrs et al 2001). Therefore, spines can both emerge directly from the dendritic shaft or from the maturation of filopodial protrusions.

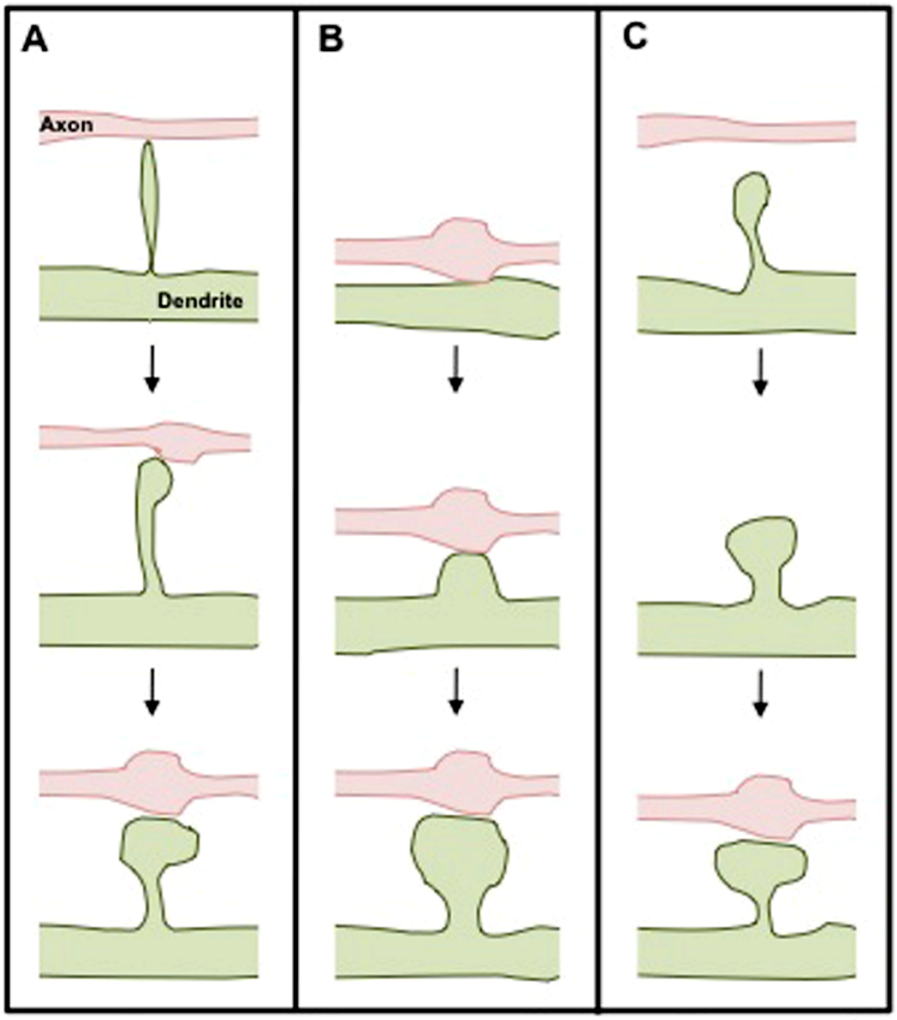



**Figure 1.2: Three different models for spine formation.** (A) According to the "filopodial model" dendritic filopodia sense the local environment for an appropriate target. Once an axo-dendritic contact is established, filopodia mature into dendritic spines. (B) The "Milller-Peters model" describes that a stubby spine can arise from a shaft synapse and then get converted into a mature mushroom type spine. (C) In the "Sotelo model" axo-dendritic contact is not required for the formation of dendritic spines.

### Spine formation without synaptic contact (Sotelo model)

In the two models discussed above, which are based on studies of pyramidal neurons, the formation of synaptic contacts triggers spinogenesis. In contrast, in

cerebellar Purkinje neurons the formation of spine-like protrusions is not depended on axonal contact (Sotelo 1990, Takacs et al 1997), but instead depends on the intrinsic and not well-understood property of the Purkinje cell. Intriguingly, Purkinje cells form spines even in the absence of innervation, since mutant mice that lack granule cells - the presynaptic partners of Purkinje cells – show normal spine development (Sotelo 1975). A more recent study reported that this model applies also for the barrel cortex, as EM reconstruction experiments revealed that spine growth precedes synapse formation (Knott et al 2006).

In summary, different mechanisms have been proposed for the formation of dendritic spines. However, it is currently unclear whether these mechanisms are intrinsic to the neuron, or whether they preferentially occur during different developmental stages or whether these models describe three different processes that happen simultaneously during postsynaptic differentiation.

### 1.3.3 Synaptogenic molecules

Over the past decade a vast number of membrane-bound and secreted signalling molecules have been identified to regulate synapse formation and maturation in the central nervous system. Importantly, some of these molecules can trigger bi-directional signalling to induce the coordinated assembly of presynaptic terminals and postsynaptic sides. The initial stabilisation of axo-dendritic contacts appears to be mediated by trans-synaptic adhesion proteins. This class of synaptogenic factors includes the classic cell adhesion molecules Cadherins and members of the immunoglobulin (Ig) superfamilies including Neurexins (NRX), Neuroligins (NLNG), Synaptic cell adhesion molecules (SynCAMs), Synaptic adhesion-like molecules (SALMs), Ephrins and Signal regulatory proteins (SIRPs) (Anggono & Huganir 2012, Benson & Huntley 2012, Gerrow & El-Husseini 2006, Han & Kim 2008, Klein 2009, McMahon & Diaz 2011, Siddiqui & Craig 2011, Toth et al 2013). In addition to trans-synaptic adhesion molecules, secreted signalling molecules are also crucial for synaptogenesis. These include the Brain-Derived Neurotrophic Factor (BDNF), members of the Wnt and FGF family, the morphogen Sonic hedgehog, the glia-derived factors Thrombospondins and the axon-guidance molecules Semaphorins (Budnik & Salinas 2011, Johnson-Venkatesh & Umemori 2010, Koropouli & Kolodkin 2014, Mitchell et al 2012, Park

& Poo 2013, Risher & Eroglu 2012, Sasaki et al 2010). Here I provide a brief overview of the role of the membrane-bound and secreted molecules implicated in central synaptogenesis. Since this thesis examines the role of Wnt signalling in actin dynamics during synapse formation, Wnt signalling factors will be discussed extensively in Section 1.6.

**Membrane-bound synaptogenic molecules**

❖ **Cadherins**

Cadherins are a large family of cell adhesion proteins with over 100 members that bind to α- and ß-catenin, which in turn bind to the actin cytoskeleton. Several Cadherins have been implicated in both excitatory and inhibitory synapse formation and maturation (Arikkath & Reichardt 2008, Benson & Huntley 2012, Brigidi & Bamji 2011, Paradis et al 2007). The most studied family member is N-cadherin, which is localized in both excitatory and inhibitory synapses in young neurons, but later is restricted to excitatory synapses (Benson & Tanaka 1998, Xie et al 2008). Extensive evidence has revealed that N-cadherin regulates the assembly of both pre- and postsynaptic sides by promoting the recruitment of several synaptic components (Arikkath & Reichardt 2008, Brigidi & Bamji 2011). Importantly, blockage of N-cadherin leads to the formation of immature spines (Togashi et al 2002), indicating that N-cadherin mediated adhesion is required for synapse maturation. However, conditional KO mice to N-cadherin, where N-cadherin gene was deleted postnatally, exhibit no changes in spine morphogenesis or basal synaptic transmission, but they have profound defects in structural and functional plasticity (Bozdagi et al 2010). These studies suggest that N-cadherin is important for activity-dependent synapse formation and strengthening *in vivo*, but not for initial synapse formation and stabilization.

❖ **Neurexins /Neuroligins**

Neurexin (NRX) and Neuroligin (NLGN) are heterophilic trans-synaptic adhesion proteins that have important roles in synapse formation. NRX is located presynaptically and binds to the postsynaptic NL in a $Ca^{2+}$-dependent manner (Craig et al 2006, Ichtchenko et al 1996, Nguyen & Sudhof 1997). There are three Neurexin genes in mammals (*NRXN1, NRXN2* and *NRXN3*), each having two alternative promoters that drive the expression of the long ⟨-neurexins and a

downstream promoter that generates the short β-neurexins (Tabuchi & Sudhof 2002). In addition, Neurexins have 5 different splicing sites resulting in the formation of up to 2000 potential variants (Missler & Sudhof 1998, Rowen et al 2002, Tabuchi & Sudhof 2002). The postsynaptic Neuroligins are expressed through five different genes (*NLGN1, NLGN2, NLGN3, NLGN4* and *NLGN4Y*), which also get alternative spliced in 2 sites. Thus, NRX and NLGN represent the most diverse family of synaptogenic molecules until now.

The extensive alternative splicing of both NRXs and NLGNs has been implicated in their function in excitatory and inhibitory synapse formation. The first indication that the NRX/NL complex is involved in synapse formation emerged from co-cultures of neurons within heterologous cells, where gain of function of NL or α-NRX promoted clustering of pre- and postsynaptic proteins, respectively (Graf et al 2004, Sabo et al 2006, Scheiffele et al 2000). Interestingly, NL1 mutant mice show a defect in glutamatergic synaptic transmission, whereas NL2 mutant mice show defects in GABAergic neurotransmission (Chubykin et al 2007). Similarly, β-NRXs promote the clustering of both excitatory and inhibitory postsynaptic components (Graf et al 2004), whereas α-NRXs induce the formation GABAergic synapses without affecting excitatory synapses (Kang et al 2008). Thus, specific NRX/NL interactions can control the balance between excitatory to inhibitory synapse formation (or excitation and inhibition). However, gain of function NL1 promotes the formation of synapses that lack AMPARs, also known as “silent synapses” (Sara et al 2005). This finding indicates that NRX/NL are not sufficient to assemble fully functional synapses, but promote the initial stages of synapse development.

Although the role of Neurexins and Neuroligins in synapse formation had been well established *in vitro*, studies using 〈-neurexin KO mice or NL1;NL2;NL3 triple knockout mice revealed no significant differences in synapse number and ultrastructure (Dudanova et al 2007, Missler et al 2003, Varoqueaux et al 2006), thus demonstrating that Neurexins and Neuroligins are not required for synapse formation *in vivo*. However, an elegant recent study showed that synapse formation does not depend to the actual levels of NL1 in each cell, but on the transcellular differences in NL1 levels between neurons (Kwon et al 2012). Therefore, these finding suggest that, at least in the case of the developing cortex, neurons compete with each other to form contacts and NL1 signalling is

required for this process. It remains to be examined whether Neurexins are also involved in synapse formation based on transcellular competition.

❖ **Synaptic cell adhesion molecules (SynCAMs)**

SynCAMs (SynCAM1-4) are also heterophilic trans-synaptic cell adhesion molecules that promote the formation of functionally releasing sites in contacting axons when expressed in heterologous cells (Biederer et al 2002). However, gain of function of SynCAM1 in young neurons enhances presynaptic function by increasing mEPSC frequency and the number of recycling synaptic vesicles, without affecting the number of synapses *in vitro* (Fogel et al 2007, Sara et al 2005). These results indicate that SynCAM1 promotes the maturation of synapses rather than the formation of new synapses (Sara et al 2005). However, *in vivo* studies revealed that elevated expression of SynCAM1 promotes the formation of excitatory synapses without affecting the number of inhibitory synapses (Robbins et al 2010). Importantly, KO mice for SynCAM1 have fewer excitatory synapses (Robbins et al 2010). The differences between the *in vitro* and *in vivo* studies can be explained by the different methods used to quantify synapses. In the Sara et al. study synapses where quantified based on the number of dendritic spines and the density of Synapsin puncta (Sara et al 2005), which is not a specific marker for excitatory synapses. In contrast, in the Robbins et al examined synapses by quantifying at the EM level (Robbins et al 2010). These findings suggest that SynCAM1 regulates the formation of excitatory synapses located on dendritic shafts or that SynCAM1 promotes the innervation of existing spines, without promoting the formation of new postsynaptic sides.

❖ **Synaptic adhesion-like molecules (SALMs)**

SALMs (SALM1-5) represent a recently identified class of cell adhesion molecules that are found at glutamatergic synapses and induce postsynaptic differentiation (Ko et al 2006, Wang et al 2006). Gain of function of SALM1 induces clustering of postsynaptic components (Wang et al 2006), whereas gain of function of SALM2 induces spine formation (Ko et al 2006). Conversely, knockdown of SALM2 reduces the number of excitatory synapses and dendritic spines, as well as the frequency, but not amplitude, of mEPSCs (Ko et al 2006), suggesting that SALM2 is not important for synaptic strength. Interestingly, a recent study showed that both SALM3 and SALM5 promote the clustering of both excitatory and inhibitory

presynaptic markers, whereas only SALM5 induced the clustering of PSD95 (Mah et al 2010). However, the ligands of SALM3 and SALM5 or their subcellular distribution have not been identified yet. Altogether these findings demonstrate that members of the SALM family can promote the co-ordinated assembly of both excitatory and inhibitory synapses.

❖ **Ephs/Ephrins**

Eph receptors (EphA1-A8 and EphB1-B4 & B6) are tyrosine kinase receptors (RTKs) that are activated by members of the ephrin family (ephrin A1-A5 and B1-B3) and are involved in both synapse formation and maturation. Ephrin-As are GPI-linked molecules and promote forward signalling upon binding to EphA receptors, whereas binding of the transmembrane ephrin-Bs to EphB receptors results to both forward and reverse signalling, thus inducing bi-directional signalling (Klein 2009).

The best-studied molecules are the ephrin-Bs and EphBs. EphB2 expression in heterologous cells induces functional presynaptic terminals in axons of contacting neurons (Kayser et al 2006). EphB2-dependent presynaptic formation is mediated through binding to ephrin-B1 or ephrin-B2, but not ephrin-B3, as shRNA-mediated silencing of ephrin-B1 or ephrin-B2 abolishes the EphB2-mediated presynaptic clustering (McClelland et al 2009). Importantly, expression of mutant ephrin-B1 that lacks the cytoplasmic domain results in a reduced number of excitatory synapses *in vivo* (Lim et al 2008), whereas infusion of the extracellular domain of EphB2 – that promotes reverse signalling through ephrinBs - increases the number of functional presynaptic sites, without affecting postsynaptic differentiation (Lim et al 2008). However, mice lacking EphB2 or conditional knock out mice to ephrin-B2 show no changes in synapse numbers (Grunwald et al 2004, Grunwald et al 2001), suggesting that ephrinB/EphB signalling is not required for synapse formation *in vivo*.

In contrast to ephrin-B1 and ephrin-B2, ephrin-B3 is localised postsynaptically (Grunwald et al 2004, McClelland et al 2010, Rodenas-Ruano et al 2006, Xu et al 2011). However, its role on synapse formation is unclear, as different groups using the same ephrin-B3 mutants have shown that loss of ephrin-B3 reduces or has no effect or increases the number of excitatory synapses in CA1 area (Aoto et al 2007, Rodenas-Ruano et al 2006, Xu et al 2011). Interestingly, in cortical

neurons ephrin-B3 signalling promotes cellular competition, as synapse density is correlated with the relative ephrin-B3 expression levels between neurons (McClelland et al 2010), similar to the effects observed with NL1 (Kwon et al 2012). Therefore, ephrin-B3 seems to play different roles in the regulation of synapse formation in different types of neurons. The fact that ephrin-B3 induces a mechanism that offers cellular competition between neurons (McClelland et al 2010) suggests that the lack of an effect in synapse density by loss of function of EphB2 *in vivo* (Grunwald et al 2004, Grunwald et al 2001) could be explained on this basis, as heterogenotypic culture experiments showed that the presynaptic ligand of ephrin-B3 is EphB2 (McClelland et al 2010).

Ephrin-B/EphB signalling is also important for synaptic function and synaptic plasticity. Application of soluble forms of ephrin-Bs or perfusion of postsynaptic neurons with peptides and antibodies that block the binding of EphB receptors to postsynaptic scaffold proteins results in impaired LTP at mossy fiber-CA3 synapses (Contractor et al 2002). In addition, mice lacking the EphB2 receptor show impairments in both LTP and LTD (Grunwald et al 2001). Furthermore, transgenic mice expressing a mutant ephrin-B3 that lucks its intracellular domain or ephrin-B3 null mutant mice or conditional knockout mice to ephrin-B2 show defects in LTP (Armstrong et al 2006, Grunwald et al 2004, Rodenas-Ruano et al 2006). Ephrin-B3 and B2 are also required for normal expression of LTD, as null mutant mice for ephrin-B3 or conditional knockout mice to ephrin-B2 show strong LTD defects (Grunwald et al 2004). Therefore, signalling by member of the ephrin-B and EphB family plays important roles in synaptic plasticity.

The role of ephrin-As and EphAs in synapse formation and plasticity is less studied. Up to date, only the role of ephrin-A5 and EphA4 has been reported. Loss of function of Ephrin-A5 leads to impaired spine morphogenesis *in vivo*, as ephrin-A5 null mice have more filopodia-like protrusions and fewer dendritic spines in the cortex (Guellmar et al 2009). EphA4 knockout mice or slices transfected with a kinase-dead EphA4 show impairments in spine morphogenesis in the hippocampus (Murai et al 2003) and defects in LTP in CA3-CA1 synapses (Filosa et al 2009, Grunwald et al 2004).

❖ **Signal regulatory proteins (SIRPs)**

SIRPs ($\alpha$, $\beta$ and $\gamma$) are transmembrane molecules that belong to the immunoglobulin superfamily. SIRPs were recently identified as postsynaptically located synaptogenic factors in the CNS (Toth et al 2013). Interestingly, neuronal activity triggers the cleavage of the extracellular domain of SIRP$\alpha$ via signalling through CaMK and matrix metalloproteases. The cleaved domain acts on the presynaptic terminal and promotes clustering of presynaptic components (Toth et al 2013). Importantly, SIRPα KO mice show defects on basal transmission, neurotransmitter release LTP, demonstrating that SIRPα is important for both synaptic function and plasticity *in vivo*.

## Secreted synaptogenic molecules

❖ **Brain-derived neurotrophic factor (BDNF)**

BDNF is a pan-synaptogenic Neurotrophin that promotes the formation of both excitatory and inhibitory synapses (Park & Poo 2013, Vicario-Abejon et al 1998). Importantly, BDNF also induces AMPAR delivery at synapses (Caldeira et al 2007), whereas blockage of presynaptic BDNF signalling impairs HFS-induced synaptic potentiation (Jia et al 2010, Shen et al 2006). Thus, BDNF not only promotes synapse formation, but activity-induced secretion of BDNF at synapses could promote synapse maturation and facilitates synaptic transmission. However, BDNF knockout mice do not exhibit any significant defect in excitatory synapse number (Itami et al 2003, Korte et al 1995, Patterson et al 1996), although mutant mice to the BDNF receptor, TrkB, show a decrease in excitatory synapse density (Luikart et al 2005, Martinez et al 1998). In contrast, mice that overexpress BDNF show accelerated GABAergic innervation and inhibition in the developing visual cortex (Huang et al 1999). These findings suggest that BDNF is not required *in vivo* for synapse formation and another Neurotrophin (eg. NT-3) acting through the TrkB receptor can compensate synaptogenesis in the absence of BDNF.

❖ **Fibroblast growth factors (FGFs)**

FGFs are a large family of growth factors that signal through four receptors (FGF receptor 1-4) consisting of three immunoglobulin-like domains, a single transmembrane domain, and a cytoplasmic tail with tyrosine kinase activity.

FGF2, which is the most highly expressed family member in brain (Reuss & von Bohlen und Halbach 2003), promotes the formation of glutamatergic synapses in hippocampal cultures, as revealed by the apposition of Synapsin1 and Synaptophysin to PSD95 and the AMPAR subunit GluA1 (Li et al 2002). Importantly, elevated expression of FGF2 *in vivo* increases the number of glutamatergic synapses, without affecting GABAegic synapse formation (Zucchini et al 2008), thus affecting the balance in excitation/inhibition. Indeed, these mice show increased susceptibility to kainate-induced seizures (Zucchini et al 2008). In addition to FGF2, FGF4, FGF6, FGF7, FGF9, FGF10 and FGF22 also induce Synapsin1 clustering (Umemori et al 2004). Most importantly, this same study provided evidence that FGF22 is secreted from granule cells of the developing mouse cerebellum *in vivo* and acts through FGFR2 on incoming mossy fibres to promote presynaptic differentiation, as assayed through observing the clustering of several presynaptic proteins (Umemori et al 2004). Furthermore, mice deficient to FGF7 and FGF22 show defects in the clustering of presynaptic inhibitory and excitatory markers, respectively, without affecting the clustering of postsynaptic components in the hippocampus (Terauchi et al 2010). Thus, the role of FGFs as target-derived presynaptic organisers is well established. Interestingly, exogenous expression of FGF7 showed a profound co-localization with the postsynaptic inhibitory marker Gephyrin, whereas expression of FGF22 showed co-localization with the excitatory postsynaptic marker PSD95 (Terauchi et al 2010). This finding suggests that the differential effect of FGF7 and FGF22 different types of synapses depend on the localisation of their receptors.

- **Thrombospondins (TSPs)**

Thrombospondins (TSP1-5) are components of the extracellular matrix made by glial cells that promote synapse formation (Adams et al 2001, Christopherson et al 2005, Eroglu et al 2009). Application of any TBP isoform to cultured retinal ganglion cells increases the number of glutamatergic synapses (Christopherson et al 2005, Eroglu et al 2009), without affecting GABAergic synapses (Hughes et al 2010). Importantly, TSP1 and TSP2 are required for synaptogenesis *in vivo*, as *TSP1*;*TSP2* double knockout mice exhibit defects in synapse numbers in the cortex (Christopherson et al 2005). However, the new synapses induced by TSP1 are silent, as they lack AMPARs (Christopherson et al 2005), demonstrating that TSP1 is not sufficient to induce the formation of mature synapses. Interestingly,

conditioned-media from astrocytes promotes the formation of both excitatory and inhibitory synapses that are not silenced (Christopherson et al 2005, Hughes et al 2010). These findings indicate that astrocytes secrete another factor(s) that promotes the unsilencing and maturation of excitatory synapses and the formation of inhibitory synapses.

❖ **Semaphorins (Sema)**

Semaphorins (Sema3A-F, 4A-G, 5A-B, 6A-D, and 7A) are axon-guidance secreted and membrane-bound molecules that play important roles in synapse formation (Koropouli & Kolodkin 2014, Shen & Cowan 2010, Yoshida 2012). In particular, shRNA-mediated silencing of Sema4B impairs both glutamatergic and GABAergic synapse formation, whereas knockdown of Sema4D specifically impairs specifically GABAergic synapse formation (Paradis et al 2007). In contrast, gain of function of Sema3A and Sema5B reduces synapse density, whereas Sema5B knockdown has the opposite effect (Bouzioukh et al 2006, O'Connor et al 2009). Interestingly, mutant mice to the Semaphorin receptor Neuropilin 2 exhibit an increase in dendritic spine density and growth in the hippocampus (Gant et al 2009, Tran et al 2009). Consistently, mice null to Sema3F or its receptor PlexinA3 show an increase in both spine number and growth in the hippocampus and cortex (Tran et al 2009). Thus, different members of the Sema family can either promote or inhibit glutamatergic synapse formation. It remains to be determined which are the molecular pathways that are activated downstream of Semaphorins to produce these distinct outcomes in glutamatergic synapses.

❖ **Sonic Hedgehog (Shh)**

Sonic Hedgehog is a very-well characterised morphogene that plays crucial role in embryonic development, including the patterning of the CNS (Cohen et al 2013, Fuccillo et al 2006). However, expression of Shh and its receptors Patched and Smoothened in postnatal brain (Petralia et al 2011a, Petralia et al 2011b, Sasaki et al 2010, Traiffort et al 1999), suggested that Shh is also involved in neuronal circuit formation and/or function in the adult brain. Indeed, a recent study showed that Shh induces the formation of large presynaptic excitatory and inhibitory terminals and promotes excitatory neurotransmitter release, without affecting the clustering of postsynaptic components (Mitchell et al 2012). In contrary, another study showed that Shh through the Smoothened receptor

promotes the formation of dendritic spines (Sasaki et al 2010). However, blockade of endogenous Shh signalling by a specific antagonist of its receptor Smoothened showed no changes in presynaptic assembly (Mitchell et al 2012), suggesting that either Shh acts through a different receptor or that Shh signalling is not required for synapse assembly.

In summary, the formation of synaptic connections requires the differentiation of both pre- and postsynaptic sides. This process is tightly regulated by a variety of transynaptic molecules and secreted factors that act as synaptic organizers. Several molecules have been implicated in the co-ordinated assembly of pre- and postsynaptic sides, whereas others affect synapse maturation rather than formation. Interestingly, some of these synaptic organisers has been shown to regulate both excitatory and inhibitory synapse development (pan-synaptogenic factors), whereas other promote specifically the formation of a specific type of synapses, thus affecting the balance of excitatory/inhibitory inputs in a neuronal circuit.

## 1.4 Synapse plasticity

After initial synapse formation and stabilization, synapses undergo functional and morphological changes in response to neuronal activity. Elevated neuronal activity results in synapse strengthening and the formation of new synapses, whereas a low neuronal activity leads to synapse weakening and synapse elimination. This dynamic property of synapses was first introduced in the 1940s by the Canadian scientist Donald Hebb who describe that *"When an axon of cell A is near enough to excite a cell B and repeatedly or persistently takes part in firing it, some growth process or metabolic change takes place in one or both cells such that A's efficiency, as one of the cells firing B, is increased."* (Hebb, 1949; for the life and work of Dolnald Hebb, see (Brown & Milner 2003)). The Hebbian theory was demonstrated several years later by Tim Bliss and Terje Lomo (1973) who found that tetanic stimulation of DG-GC synapses in the hippocampus of anesthetized rabbits leads to a long-lasting enhancement in synaptic efficacy (Bliss & Lomo 1973). Soon after, the term long-term potentiation (LTP) and long-term depression (LTD) were introduced to describe any long-lasting (> 1 hr), activity-induced change in synaptic strength.

Currently, long-term potentiation (LTP) and long-term depression (LTD) are the most established models - induced by electrical or chemical stimuli - to study synaptic plasticity. LTP and LTD are expressed through both presynaptic and postsynaptic mechanisms via changes in neurotransmitter release, channel conductance and surface localisation of glutamatergic receptors, gene expression and modifications of the actin cytoskeleton (Bliss & Collingridge 2013, Bosch & Hayashi 2011, Bramham 2008, Coultrap & Bayer 2012, Kerchner & Nicoll 2008, Kessels & Malinow 2009, Lee & Kirkwood 2011, Nicoll & Roche 2013).

### 1.4.1 LTP and LTD expression

It is widely accepted that both LTP and LTD are mainly, but not exclusively, NMDA receptor-depended (Bliss & Collingridge 2013). Although other forms of LTP and LTD have been also described, including mGluR-dependent LTD and Endocannabinoid-mediated LTD (Kauer & Malenka 2007, Raymond 2007), these types are not going to be discussed here for simplicity. Under basal conditions AMPARs are active, whereas the channel pore of NMDARs is blocked by $Mg^{2+}$. Binding of glutamate to AMPARs leads to depolarization of the postsynaptic membrane, which relieves the $Mg^{2+}$ blockage of NMDARs and results in $Ca^{2+}$ influx into the postsynaptic side (Nicoll et al 1988), which then initiates a cascade of signalling events.

An extensive debate that lasts for several years in the field of synaptic plasticity is whether LTP and LTD are pre- or postsynaptically expressed (Bliss & Collingridge 2013, Nicoll & Roche 2013). The most compelling evidence that supports a postsynaptic mechanism came from the development of uncagging experiments. Repetitive uncagging of glutamate in single spines, which activates locally NMDARs and AMPARs overpassing the requirement of a realising presynaptic site, increases the AMPAR-mediated response of that same spine as well as spine enlargement (Matsuzaki et al 2004). Importantly, the effect in synaptic efficacy evoked by glutamate uncaging is similar to the one elicited by electrical stimulation (Harvey & Svoboda 2007). These findings demonstrate that at least in hippocampal synapses the presynaptic component is not required to induce LTP. Given that LTP is postsynaptically expressed, the modifications seen in neurotransmitter release will require an activity-induced retrograde messenger, capable to act on the presynaptic terminal. Several molecules have been demonstrated to act as activity-dependent retrograde messengers,

including nitric oxide (NO) signalling (Feil & Kleppisch 2008) and activity-induced cleavage of SIRPs (Toth et al 2013).

The precise molecular pathways that are activated by neuronal activity to facilitate changes in synaptic efficacy remain poorly understood. Although the mechanism that modulates the unsilencing of synapses – those that lack AMPARs – is better understood as well as the modulation of functional properties of AMPARs (Kessels & Malinow 2009, Lee & Kirkwood 2011, Opazo et al 2012). Upon LTP, silent synapses became unsilenced by the insertion of AMPARs (Kerchner & Nicoll 2008), leading to an increase in the frequency of AMPAR-mediated mEPSCs. In addition to synapse unsilencing, synapses become stronger upon LTP due to modifications in the functional properties of AMPARs, leading to an increase in the amplitude of AMPAR-mediated mEPSCs. These changes include modifications in channel opening probability, decay time and channel conductance.

### 1.4.2 AMPAR regulation upon LTP and LTD

The most well characterised mechanism to increase AMPAR conductance is through phosphorylation of the GluA1 subunit by the $Ca^{2+}$/calmodulin-dependent protein kinase II (CaMKII) and protein kinase C (PKC) on Ser831 (Barria et al 1997, Roche et al 1996). GluA1 has three additional phosphorylation sites at Ser818, Thr840 and ThrS845 (Lu & Roche 2012). Phosphorylation on Ser818 by PKC is required for LTP, whereas phosphorylation on Thr840 has been implicated in LTD. Finally, Ser845 which is phosphorylated by PKA regulates its opening probability.

In addition to changes in AMPAR conductance and opening probability, LTP and LTD modulate AMPAR trafficking. AMPARs are now well-known to dynamically traffic in and out from the plasma membrane and from extrasynaptic sites to the PSD (Choquet 2010, Opazo et al 2012). This trafficking is highly regulated during LTP and LTD resulting in changes in the number of surface synaptic receptors. Upon LTP, receptors from extrasynaptic sites are moving laterally to synapses resulting to synaptic strengthening (Hayashi et al 2000, Shi et al 1999), whereas during LTD these receptors diffuse away from the synapse and are subsequently endocytosed resulting in synaptic weakening (Beattie et al 2000, Carroll et al

1999). These results demonstrate that AMPARs are trafficking in and out of the synapse in an activity-dependent manner.

AMPAR subunit composition has been also implicated in synaptic plasticity. AMPARs are hetero-tetrameric composed of four types of subunits (GluA1-4), which first assemble in the endoplasmic reticulum (ER) as dimers, which come together to form "dimers of dimers" and make a tetramer. In adult rat hippocampal neurons AMPARs are predominantly composed of dimers of GluA1/2 or GluA2/3 subunits, whereas synaptic AMPARs are mainly combinations of GluA1 and GluA2 (Henley & Wilkinson 2013). GluA2-containing AMPARs are $Ca^{2+}$ impermeable, whereas GluA2-lacking - most likely GluA1 homomers – are $Ca^{2+}$ permeable and are those that have been implicated in LTP. AMPAR delivery to synapses upon LTP requires the GluA1 subunit and its binding to proteins in the PSD (Hayashi et al 2000, Shi et al 2001), whereas LTD-mediated AMPAR endocytosis dependents on PSD binding of the GluA2 subunit and its phosphorylation (Ahmadian et al 2004, Shi et al 2001). In addition, it has been suggested that upon LTP GluA1 homomers, thus $Ca^{2+}$ permeable, are initially recruited to synapses and subsequently get replaced by GluA2-containing ($Ca^{2+}$ impermeable) receptors (Jaafari et al 2012, Plant et al 2006). These results support the notion that LTP and LTD expression depends on the presence of specific AMPAR subunits.

In conclusion, since Hebb proposed a model for synapse plasticity (Hebb, 1949; for the life and work of Dolnald Hebb, see (Brown & Milner 2003)), great progress been made in understanding how neuronal circuits adapt to patterns of neuronal activity. Until now, we have discovered that LTP and LTD are mainly postsynaptically expressed and highly-dependent on AMPAR functional properties and membrane trafficking into the synapse. However, although extensive studies have been performed to elucidate the mechanisms that lead to LTP and LTD expression, the molecular mechanisms that lead to synapse potentiation or synapse depression need further examination.

# 1.5 Actin cytoskeleton in excitatory synapse formation & plasticity

The actin cytoskeleton is a fundamental regulator of cellular morphology, from neurons/glia to small structures such as synapses. In particular, the actin cytoskeleton is the underlying structural brick of synapses and is involved in a variety of processes from organizing the presynaptic and postsynaptic terminals to anchoring of postsynaptic receptors and signalling molecules (Cingolani & Goda 2008, Frost et al 2010, Goellner & Aberle 2012, Hotulainen & Hoogenraad 2010, Menna et al 2011). In this part of the introductory chapter, I focus on the role of actin dynamics in axon remodelling and in the formation of synapses, in particular on our current understanding of the role of actin-capping proteins in the regulation growth cone remodelling, filopodia and spine formation.

## 1.5.1 Actin organisation in neurons

Actin exists in two forms: monomeric globular actin (G-actin) and filamentous actin (F-actin), a two-stranded asymmetrical helical polarised polymer, which is formed via weak non-covalent interactions of G-actin. At steady-state, F-actin polymerizes at one end of the filament (plus or barbed end), while G-actin monomers are disassembled from the other end (minus or pointed end) (Figure 1.3A). The difference in polymerization rates between the two ends of the filament result in a continuous turnover of filaments, a process called treadmilling.

In migrating cells, the barbed ends of actin filaments face the plasma membrane and form flat mesh-like and spike-like protrusions, called lamellipodia and filopodia, respectively (Pollard & Borisy 2003). Lamellipodia are composed from a meshwork of actin filaments, whereas filopodia are composed of bundles of long and linear F-actin. A wide range of actin-binding proteins (ABPs) can influence actin organisation and dynamics in different ways, resulting in the formation of lamellipodia and filopodia (Figure 1.3B). For example, some ABPs act as bundling proteins (e.g. Fascin) by guiding the formation of tight bundles of F-actin, resulting in high structural strength that is required to overcome the force applied from the cellular membrane (Mogilner & Rubinstein 2005). Other ABPs affect F-actin turnover by severing filaments (e.g., Gelsolin and Cofilin) or sequestering actin monomers (e.g. Profilin) or by capping either the barbed end

(e.g. CP and Eps8) or the pointed end (e.g. Tropomodulin) of actin filaments (Figure 1.3A).

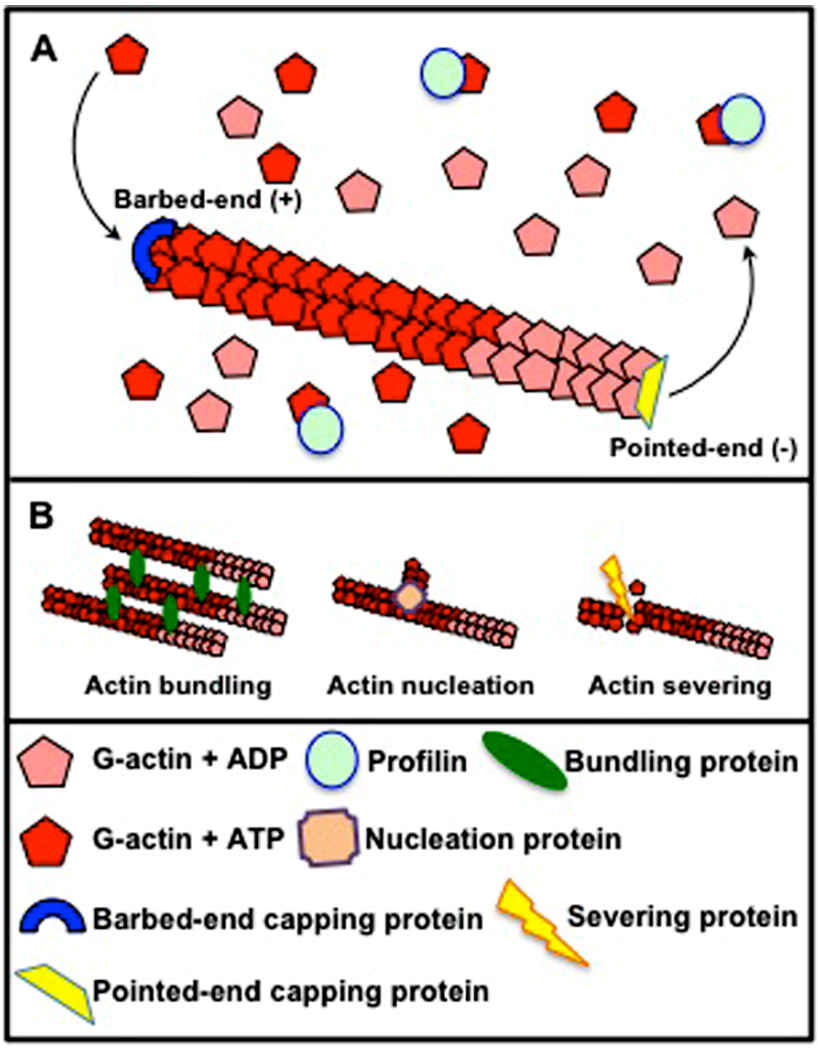



**Figure 1.3: Actin cytoskeleton and organisation.** (A) Filamentous actin is a double-stranded polymer composed of monomeric actin (G-actin). F-actin polymerizes at one end of the filament (barbed end), whereas it dissembles from the other end (pointed end). Proteins that bind to filament ends (capping proteins) inhibit the assembly and disassembly of actin monomers. (B) Actin organisation is regulated by actin-binding proteins, such as actin-bundles, actin-nucleators and severing factors.

In neurons, F-actin is the most dominant cytoskeletal protein and is present in the both axons and dendrites. In particular, actin is highly enriched in growth cones and dendritic spines (Korobova & Svitkina 2010). The axonal growth cone can be divided into three distinct regions according to the organisation of the actin filaments: the peripheral and central domain and the transition zone (Figure 1.4A). In the peripheral domain actin is organised into linear actin bundles (filopodia) and mesh-like networks (lamellipodia). The central domain

devoid F-actin but is enriched in microtubules, whereas the transition zone is characterised by the presence of bundles of actin filaments aligned in parallel to the leading edge, which are called actin arcs (Fig. 1.4A). In mature spines, the neck is formed by linear F-actin, whereas the spine head by branched actin filaments, respectively (Figure 1.4B) (Korobova & Svitkina 2010). Presynaptic boutons also contain a very similar branched network to spine heads. Thus, axonal growth cones, presynaptic boutons and spine heads are composed from mesh-like actin networks similar to those observed at the leading edge of migrating cells. In contrast, neuronal filopodia and spine necks consist of long linear actin bundles similar to those found in conventional filopodia.

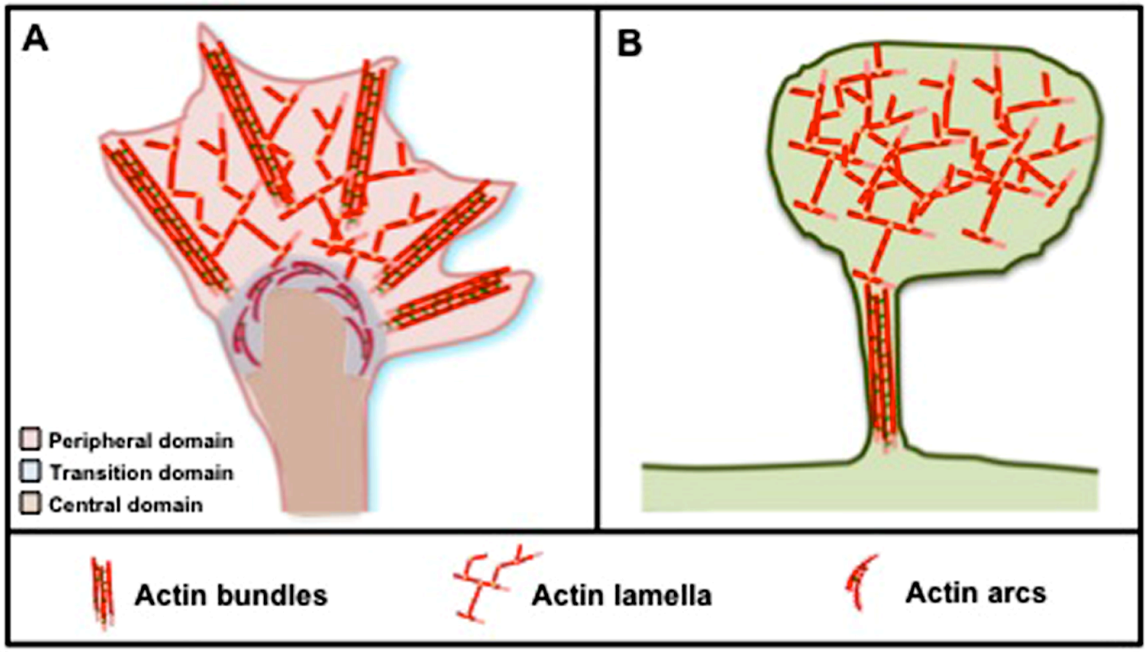



**Figure 1.4: Growth cones and dendritic spines are highly enriched in actin.** (A) Growth cones are subdivided into 3 regions based on the organisation of the actin cytoskeleton. The peripheral domain consists of actin bundles and mesh-like actin network. The transition zone has actin bundles aligned parallel to the leading edge, whereas the central domain avoids actin. (B) Dendritic spine heads are composed by actin lamellas, whereas spine necks contain actin bundles similar of those observed in filopodia.

## 1.5.2 Filopodium formation: focus on actin-capping proteins

It is widely accepted that filopodia play important roles during the initial stages of synaptogenesis (Craig et al 2006, Fiala et al 1998, Geraldo & Gordon-Weeks 2009). Axonal filopodia emerging from growth cones mediate axonal steering (Dent et al 2011, Geraldo & Gordon-Weeks 2009, Lowery & Van Vactor 2009), whereas those emerging from the axonal shaft may represent precursors of presynaptic boutons (Matteoli et al 2004). In contrast, dendritic filopodia are

considered as precursors of dendritic spines (Ethell & Pasquale 2005, Yuste & Bonhoeffer 2004). Thus, the molecular mechanisms that regulate filopodium initiation in neurons are of particular importance in the formation of neuronal circuits.

The most accepted model for filopodium formation starts with the elongation of F-actin within a lamellipodium. Several actin-binding proteins have been implicated in regulating the balance between filopodia and lamellipodia (Gupton & Gertler 2007, Le Clainche & Carlier 2008, Ridley 2011). The formation of lamellipodia mainly involves activation of the Arp2/3 complex (Hotulainen et al 2009), an actin filament nucleator, that functions in collaboration with actin-capping proteins (Akin & Mullins 2008). Capping proteins bind to the barbed end of actin filaments, inhibit their elongation and promote their stabilisation (Mogilner & Rubinstein 2005). Filopodial initiation on the other hand, requires the combinatory activities of actin elongation and anti-capping proteins, including formins and vasodilator-stimulated phosphoprotein (VASP) family proteins, which promote the formation of long filaments (Drees & Gertler 2008). These actin filaments are converged into actin bundles by bundling proteins, such as Fascin (Mattila & Lappalainen 2008, Mejillano et al 2004).

Actin-capping proteins are particularly important for controlling the length and the organization of actin cytoskeleton. Studies in non-neuronal cells have indicated that when the capping activity is high, newly branched filaments become rapidly capped resulting in the local increase in the concentration of monomeric actin (G-actin). This increase leads to a higher rate of nucleation and branching, subsequently promoting the formation of a dense and highly-branched mesh of lamellipodia. In contrast, low capping activity leads to a depletion of the G-actin pool and the formation of long actin filaments (Akin & Mullins 2008, Mejillano et al 2004). Thus, loss of an actin-capping protein favours filopodium formation and limits lamellipodium formation.

In neurons, three actin-capping proteins have been shown to control filopodium formation. Gelsolin, an actin-severing and barbed-end capping protein, which is regulated by $Ca^{2+}$, promotes the retraction of axonal filopodia (Lu et al 1997). Eps8 (EGF receptor pathway substrate 8), another capping protein that is regulated by protein–protein interactions and phosphorylation, also regulates axonal filopodium formation by inhibiting their initiation (Menna et al 2009).

More recently, it was reported that silencing of the capping protein CP increases dendritic filopodium density (Fan et al 2011). These findings demonstrate that actin-capping proteins are important regulators of filopodium formation in neurons.

### 1.5.3 From filopodium formation to synaptogenesis: a role for actin-capping and anti-capping proteins

Filopodia are thought to play an active role during synaptogenesis. This concept is particularly well-established in the case of dendritic filopodia, which upon axonal contact convert into stable spines (Craig & Woodhead 2006, Yoshihara et al 2009). Axonal filopodia are also thought to be involved in initiating synapse formation, since it has been observed that they contain synaptic vesicles and synaptic proteins (Chang & De Camilli 2001, Matteoli et al 2004), thus axonal filopodia could represent precursors of presynaptic terminals.

During the last decade great progress has been made in elucidating the mechanisms that regulate the formation of dendritic spines from filopodium maturation by changes in the underlying actin cytoskeleton (Bosch & Hayashi 2011, Bramham 2008, Cingolani & Goda 2008, Hotulainen & Hoogenraad 2010, Lin & Webb 2009, Penzes & Cahill 2012). Newly formed spines are usually thin and elongated and have a small spine head, which supports the filopodium-spine conversion model. The formation of a spine head is highly dependent on actin polymerisation and the activity of the Arp2/3 nucleation complex at the tip of the dendritic filopodium. Knockdown of Arp2/3 through shRNA-mediated silencing of the p34 subunit of the Arp2/3 complex promotes filopodia-like protrusions, while decreasing the number of spines (Hotulainen et al 2009). In addition to actin filament nucleation and polymerization, three-dimensional organization of actin filaments is required during spine head formation. Actin cross-linking proteins, such as α-actinin, are important for spine head modification and stabilization (Hoe et al 2009, Nakagawa et al 2004). The activity of the actin-severing factor Cofilin is also important for the proper morphology and stabilization of newly formed spines (Hotulainen et al 2009). Also, it has been speculated that a capping activity is also required to establish the normal shape of the spine head and inhibit the formation of irregular protrusions (Hotulainen & Hoogenraad 2010, Lin & Webb 2009). Thus, the

formation of dendritic spines requires the combinatorial action of several actin-binding proteins (Table 1.1).

Notably, actin-capping and anti-capping proteins are enriched in dendritic spines. The actin-capping protein CP localizes in dendritic spines (Fan et al 2011, Korobova & Svitkina 2010) and is required for spine morphogenesis, since its silencing leads to fewer and larger spines with irregular morphology (Fan et al 2011). In contrast, gain of function of the anti-capping protein VASP, which is enriched in spine heads, leads to spine enlargement and results in a significant increase in the amount of actin filaments and uncapped barbed ends available for actin polymerization within spines (Lin et al 2010). These findings suggest that low capping activity results in large spine heads due to an increase in F-actin content.

**Table 1.1: ABPs in filopodium and spine formation.**

| ABP | Activity | Effect on filopodia | Effect on dendritic spines | References |
|---|---|---|---|---|
| α-actinin | Bundling | Gain of function: more & longer filopodia | Gain of function: less & immature spines | (Hoe et al 2009, Nakagawa et al 2004) |
| Abp1 | Nucleation | | Gain of function: more mushroom type spines with longer necks<br>Loss of function: less mushroom type spines | (Haeckel et al 2008) |
| Arp2/3 complex | Branching, nucleation | Loss of function (p34): no effect | Loss of function (p34): less thin spines & longer dendritic protrusions | (Hotulainen et al 2009, Korobova & Svitkina 2008, Wegner et al 2008) |
| Calponin | Bundling | Not determined | Gain of function: more & longer spines | (Rami et al 2006) |
| CaMKIIß | Bundling | Not determined | Loss of function: reduced spine density & growth, longer spines | (Okamoto et al 2007) |
| CP | Capping | Loss of function: more filopodia | Loss of function: less but larger spines with irregular morphology | (Fan et al 2011) |

| ABP | Activity | Effect on filopodia | Effect on dendritic spines | References |
|---|---|---|---|---|
| α-N-catenin | Bundling | Not determined | Gain of function: more spines & larger spines<br>Loss of function: longer, immature spines with enhanced motility | (Abe et al 2004) |
| Cofilin | Severing | Loss of function: no effect | Loss of function: longer, immature spines<br>DN: decreased length of spines<br>CA: longer & smaller spines | (Hotulainen et al 2009, Shi et al 2009) |
| Drebrin | Anti-bundling | Loss of function: less filopodia | Gain of function: longer & larger spines<br>Loss of function: less spines | (Hayashi & Shirao 1999, Takahashi et al 2003) |
| Eps8 | Capping & bundling | Loss of function: more axonal filopodia | Not determined | (Menna et al 2009) |
| Formin (mDia2) | Nucleation | Loss of function: less filopodia | Loss of function: fewer thin spines, but more stubby spines | (Hotulainen et al 2009) |
| Gelsolin | Anti-bundling, capping & severing | Loss of function: more filopodia | Not determined | (Lu et al 1997) |
| Neurabin I | Bundling | Gain of function: more filopodia | Gain of function: smaller & longer spines | (Terry-Lorenzo et al 2005) |
| Neurabin II/Spinophilin | Bundling | Gain of function: longer filopodia<br>Loss of function: no effect | Loss of function: more spines | (Feng et al 2000) |
| Synaptopodin | Bundling | Not determined | Loss of function: no effect | (Vlachos et al 2009) |

### 1.5.4 Actin regulation during spine plasticity

An interesting feature of dendritic spines is that they are not static, but undergo structural changes even in the adult brain, demonstrating their plastic behaviour. Live imaging studies revealed that spine morphology can be altered by neuronal activity *in vitro* and by experience *in vivo* (Bosch & Hayashi 2011, Kasai et al 2010, Knott & Holtmaat 2008, Rochefort & Konnerth 2012, Segal 2010). Patterns of stimuli that induce long-term potentiation (LTP) cause the formation of new spines and spine head enlargement, whereas stimuli that induce long-term depression (LTD) result in spine elimination and shrinkage (Bosch & Hayashi 2011, Bourne & Harris 2007). Thus, changes in neuronal activity do not only result in synaptic potentiation, but also morphological changes on dendritic spines, a process called "structural plasticity".

Dendritic spines are highly-enriched in actin, it is therefore not surprising that spine plasticity is associated with rapid changes in the organization and dynamics of the actin cytoskeleton (Cingolani & Goda 2008). Measurements of fluorescence resonance energy transfer (FRET) between actin monomers revealed that synaptic activity rapidly changes the equilibrium between F-actin and G-actin (Okamoto et al 2004). LTP induces a shift towards more F-actin and therefore increases spine volume, whereas LTD shifts the F/G-actin equilibrium towards more G-actin and results in spine shrinkage (Okamoto et al 2004). Importantly, drugs that block actin polymerization (such as Latrunculin A) suppress LTP, whereas drugs that inhibit actin depolymerisation (such as jasplakinolide) block LTD (Chen et al 2007, Fukazawa et al 2003, Krucker et al 2000, Okamoto et al 2004, Ramachandran & Frey 2009). Thus, neuronal activity modulates actin dynamics, but in addition actin dynamics are required for changes in synaptic strength.

Time-lapse studies using photoactivatable-GFP tagged actin to monitor actin turnover within dendritic spines revealed that actin exists in at least three distinct "pools" - a dynamic pool, a stable pool, and an enlargement pool (Honkura et al 2008). Under basal conditions, the dynamic pool is the dominant one and has a fast turnover rate ($\tau$ = 40 secs) (Honkura et al 2008). The stable actin pool is concentrated in the spine head base and is thought to act as a

**Table 1.2: ABPs in activity-induced actin rearrangements.**

| ABP | Activity | Effect on synaptic plasticity | References |
|---|---|---|---|
| β-adducin | Capping | Loss of function: defects in LTP maintenance | (Rabenstein et al 2005) |
| Cofilin | Severing | DN peptide: less spine shrinkage during LTD<br>CA peptide: more spine shrinkage during LTD | (Zhou et al 2004) |
| Gelsolin | Anti-bundling, capping & severing | Loss of function: impaired LFS-induced actin turnover | (Star et al 2002) |
| IRSp53 | Bundling | Loss of function: enhanced LTP | (Sawallisch et al 2009) |
| Neurabin I | Bundling | Gain of function: inhibited LTP & enhanced LTD<br>ABD deletion mutant: increased LTP & reduced LTD | (Wu et al 2008) |
| Neurabin II/Spinophilin | Bundling | Loss of function: reduced LTD, but no effect on LTP | (Feng et al 2000) |
| Synaptopodin | Bundling | Loss of function: reduced LTP | (Vlachos et al 2009) |

platform that allows the dynamic pool to produce an expansive force on the spine membrane. Interestingly, LTP induction mediated by glutamate uncagging causes a decrease in actin turnover and the formation of a third actin pool - the enlargement pool (Honkura et al 2008). Long-term increases in spine head area require the confinement of the enlargement pool in the spine head, which is dictated by the width of the spine neck and also by the cross-linking of the enlargement pool actin filaments by CaMKII (Honkura et al 2008).

Although the role of actin cytoskeleton in structural and functional plasticity has been well studied, the precise molecular mechanisms that underlie actin

cytoskeletal rearrangements during activity-mediated changes remain largely elusive. Up to date very few actin-binding protein have been implicated in cytoskeletal changes mediated by neuronal activity (Table 1.2). However, other actin-binding proteins are regulated by neuronal activity, indicating a role in spine plasticity. The actin elongation factors Profilin I and II translocate into spines in an activity-dependent manner (Ackermann & Matus 2003, Neuhoff et al 2005). However, mice deficient to Profilin II do not show any defects in LTP or LTD (Pilo Boyl et al 2007), suggesting that Profilin I could have a redundant function during changes in neuronal activity.

In contrast to Profilin, which promotes the formation of actin filaments, Cofilin induces the severing of F-actin from the pointed end of actin filaments leading to depolymerization. In neurons, Cofilin localizes to spines and is required for normal actin turnover and spine morphogenesis (Hotulainen & Hoogenraad 2010). Phosphorylation of Cofilin at Ser3, which inhibits its severing activity, is associated with LTP-mediated spine growth and increased F-actin content (Chen et al 2007, Fedulov et al 2007, Fukazawa et al 2003), whereas dephosphorylation at Ser3 is associated with LTD-mediated spine shrinkage (Zhou et al 2004). Thus, regulation of Cofilin function is important for activity-mediated cytoskeletal reorganisation.

Two actin-capping proteins have also been implicated in synaptic plasticity. Gelsolin, an actin-capping and severing protein, is important for $Ca^{2+}$-mediated actin depolymerization in many cell types, including neurons (Furukawa et al 1997, Kinosian et al 1998). Although loss of function of Gelsolin does not affect actin turnover within spines, Gelsolin is required for actin stabilization in response to low frequency stimulation (LFS) (Star et al 2002). In addition to Gelsolin, CP (capping protein) is also probably involved in synaptic plasticity, since its expression is regulated by neuronal activity (Kitanishi et al 2010). Blockage of neuronal activity by TTX reduces CP levels, whereas HFS leads to elevated CP levels (Kitanishi et al 2010). Thus, CP could regulate actin dynamics in response to neuronal activity.

In summary, actin-binding proteins play crucial roles in regulating actin dynamics and organisation and mediated many of the structural changes induced by neuronal activity. Recent studies have indicated these proteins regulate different stages of synapse formation, from the emergence of filopodia in young

neurons to the structural changes required during activity-dependent spine formation and remodelling.

### 1.5.5 Defects in actin regulation and disease: focus on Rho small GTPases and the WAVE complex

The tight connection between spine morphogenesis with cognitive function is becoming more evident in neurodevelopmental, psychiatric and neurodegenerative disorders, where it has been observed abnormality in spine density, growth and morphology. Post-mortem studies have shown that in Alzheimer's disease there is a progressive decrease in spine density, in autism and fragile X syndrome there is an increase of spines with immature morphology, in Down's syndrome there is a decrease in spine growth, whereas in several other conditions there are abnormalities in spine shape (Fiala et al 2002). Although multiple signalling pathways are known to regulate the actin cytoskeleton in spines (Carlisle & Kennedy 2005, Ethell & Pasquale 2005, Hotulainen & Hoogenraad 2010, Penzes & Cahill 2012, Schubert & Dotti 2007, Shen & Cowan 2010, Svitkina et al 2010, Tada & Sheng 2006, Yoshida 2012), the molecular mechanisms that are affected in neurological diseases resulting in spine abnormalities are not well understood. In this section, I am introducing our current knowledge of most-well characterised actin modifying proteins that are involved in neurological diseases.

The most well studied proteins that regulate the actin cytoskeleton are the Rho family of small GTPases, which is composed of 22 members that are divided in the Rac, Cdc42 and Rho subfamilies. Small GTPases are molecular switches that are active when bound to GTP but inactive when bound to GDP. The activity of small GTPases is regulated by two classes of proteins: the guanine-nucleotide exchange factors (GEFs) which promote their activation by exchanging the GDP for GTP and the GTPase activating proteins (GAPs) which inhibit small GTPases by hydrolysing GTP to GDP (Luo 2000, Penzes & Cahill 2012).

Rac1 is the most well studied Rho family member. Karilin, which is one of the most-well characterised Rac-GEF in neurons, is highly expressed in the brain and is localised in dendrites and spines (Penzes et al 2000, Penzes et al 2001a). Upon NMDAR-mediated CamKII activation, Kalirin activates Rac1 resulting in spine formation and maturation (Penzes et al 2001b, Xie et al 2007). Mice lacking

Kalirin exhibit defects in spine formation and show schizophrenia-related behavioural phenotypes (Cahill et al 2009, Xie et al 2011, Xie et al 2008). Importantly, post-mortem studies revealed that Kalirin expression levels are decreased in brains from schizophrenia and Alzheimer's disease (AD) patients (Penzes & Cahill 2012). In addition, Kalirin directly interacts with the schizophrenia-linked protein DISC1 (Disrupted-in-Schizophrenia 1), which is enriched in postsynaptic fractions (Hayashi-Takagi et al 2010). DISC1 loss of function leads to increased size and number of spines barring functional synapses (Hayashi-Takagi et al 2010). DISC1 regulates the localisation of the Kalirin and its interaction with Rac1, therefore controlling the duration and intensity of Rac1 activation in response to NMDA receptor activation (Hayashi-Takagi et al 2010).

The Rap family (Ras-related proteins) of small GTPases is composed of two proteins, Rap1 and Rap2. Rap1/2 promotes the endocytosis of GluR2/3-containing AMPA receptors leading to spine shrinkage and synaptic depression *in vitro* and enhanced LTD *in vivo* (Ryu et al 2008, Xie et al 2005, Zhu et al 2002). Epac2 (Exchange protein activated by cAMP) is a Rap-GEF, which is highly enriched in the brain and in particular in dendritic spines and postsynaptic fractions (Woolfrey et al 2009). Similarly to Rap, Epac2 induces spine shrinkage and synapse destabilization, whereas shRNA-mediated Epac2 knockdown leads to an increase in spine growth (Woolfrey et al 2009). Mice lacking Epac2 display defects in spine formation and motility and exhibit abnormal social interactions (Srivastava et al 2012a). Importantly, Epac2 has been associated with autism (Penzes et al 2011b). Interestingly, expression of an Epac2 autism-linked variant (Epac2-G706R) in cortical neurons results in impaired dendritic arborisation, as it has been observed in patients with autism (Srivastava et al 2012b).

The WAVE regulatory complex (WRC) is another critical regulator of actin dynamics by regulating the activity the actin filament nucleator Arp2/3 (Chen et al 2010, Stradal & Scita 2006). WCR consists of five proteins: WAVE, Abi, Nap1, HSPC300 and CYFIP1. CYFIP1 (cytoplasmic FMR1 interacting protein 1) acts as an inhibitor of the WRC when the small GTPase Rac1 is inactive. Rac1 activation results in the disassociation of CYFIP1 from the WCR, which allows Arp2/3-mediated actin nucleation (Chen et al 2010, Stradal & Scita 2006). Importantly, CYFIP1 is a schizophrenia- and autism-linked protein, as alterations on its

expression levels have been associated with both conditions (Bozdagi et al 2012, Cox & Butler 2015, Wang et al 2015). Intriguingly, both deletions and duplications of the 15q11.2 locus, where the Cyfip1 gene is located, result in similar neuropsychiatric phenotypes (Bozdagi et al 2012, Cox & Butler 2015, Wang et al 2015). Gain and loss of function studies of CYFIP1 revealed that is required for dendritic arborisation and spine maturation by controlling actin dynamics and AMPAR delivery in dendritic spines (Pathania et al 2014). Interestingly, both Cyfip1 overexpression and haploinsufficiency result in defects in spine maturation both in basal and activity-induced conditions *in vitro*, whereas heterozygous mice for Cyfip1 exhibit defects in dendritic arborisation and spine maturation *in vivo* (Pathania et al 2014). These results indicate that altered levels of CYFIP1 result in actin dysregulation, leading to changes in neuronal connectivity and to neurological conditions, such as schizophrenia and autism.

In summary, a plethora of actin regulating proteins have been associated with several neurological conditions (for more detailed review on this subject see (Penzes & Cahill 2012, Penzes et al 2011a)), demonstrating that spine structural changes are highly connected to brain function. These findings provide important insights into our understanding of complex neurological diseases, which is required for therapeutic purposes. However, it remains to be determined whether the molecular mechanisms discussed above can be used for the development of drug targets.

## 1.6 Wnt signalling

Wnt signalling has been shown to modulate the formation and the function of different types of synapses. The role of Wg – the *Drosophila* Wnt homolog- in early embryonic patterning was characterised almost 40 years ago (Sharma & Chopra 1976). Since then, studies in different organisms have demonstrated a crucial role for Wnt signalling in several biological processes ranging from stem cell self-renewal to cell fate decisions, tissue polarisation and cellular homeostasis (Anastas & Moon 2013, Arwert et al 2012, Merrill 2012, Regard et al 2012, von Maltzahn et al 2012b). In the nervous system, Wnts play an important role in neuronal connectivity by regulating axon guidance, dendritic arborisation and synapse formation (Budnik & Salinas 2011, Ciani & Salinas 2005, Fradkin et

al 2005, Koles & Budnik 2012, Mulligan & Cheyette 2012, Park & Shen 2012, Salinas 2012).

### 1.6.1 Wnt proteins

Wnts are a large family (19 members in humans) of secreted palmitoylated glycoproteins (~ 40 kD) that are evolutionary conserved (Clevers & Nusse 2012). The term Wnt derives from the *Drosophila* gene *wingless* (*wg*) and the vertebrate gene *Int-1* (Nusse et al 1991). Although the amino acid sequence of Wnts has several charged residues, thus indicating efficient solubilisation in aqueous solution, isolation and characterisation of active Wnt proteins has been a challenging task. Wnt3a was the first protein that has been successfully purified on its active form (Willert et al 2003). Upon this isolation it was discovered that Wnt proteins undergo post-translational modifications that are required for their secretion and function. These modifications include N-linked glycosylation (attached to Asparagine amino acids) that is important for Wnt secretion and palmitoylation on Cystein residues that is required for both secretion and function.

Glycosylation is a post-translational modification that involves the addition of sugar to organic molecules. Glycans are important for various protein functions, such as correct folding, enzymatic activity, membrane localisation, stability or secretion. Glycosylation of Wnt3a on N87 and N298 is important for its secretion (Komekado et al 2007, Smolich et al 1993), as well as glycosylation of Wnt5a on N114, N120, N311 and N325 (Kurayoshi et al 2007). In contrast, Wnt1 has 4 potential glycosylation sites (N29, N316, N346 and N359) but none of them are important for its secretion or function (Mason et al 1992). Although initial studies reported that glycosylation of Wnt3a is a critical step for its exit from the endoplasmic reticulum (ER) and its subsequent palmitoylation (Komekado et al 2007), a recent study showed that newly synthesized non-glycosylated Wnt3a can be readily palmitoylated inside the ER, indicating that palmitoylation of Wnt3a is not dependent on its glycosylation status (Gao & Hannoush 2014). Whether glycosylation is important for the secretion of other Wnt family members remains unknown.

Palmitoylation is a type of fatty-acylation that involves the addition of a 16-carbon long acid (palmitate), resulting in an increase in protein hydrophobicity, which plays a critical role for its trafficking and membrane localisation. Palmitoylation is a very common modification of membrane-tethered and transmembrane proteins, but very unusual for secreted proteins. Several Wnt family members are palmitoylated, including Wnt3a, Wnt1, Wnt5a and Wg, on a conserved Cystein residue (C77 -referring to the sequence of the mouse Wnt3a) (Galli et al 2007, Kurayoshi et al 2007, Willert et al 2003, Zhai et al 2004). Intriguingly, palmitoylation, which increases hydrophobicity of Wnt proteins, is required for their secretion and signalling. A palmitoylation deficient Wg shows deficits on secretion (Takada et al 2006, Willert et al 2003). In contrast, C77A mutants of Wnt3a and Wnt5a are normally secreted normally, but are defective in signalling (Kurayoshi et al 2007, Smolich et al 1993, Willert et al 2003). Despite the contradictory effects of palmitoylation on Wnt3a, Wnt5a and Wg, the emerging view is that palmitoylation is important for their signalling activity.

In addition to glycosylation and palmitoylation, Wnts can undergo other post-translational modifications. For example the secretion of Wnt3a depends on the acyl-addition of palmitoleic acid - a monounsaturated fatty acid - on Ser209 (Takada et al 2006). In contrast, an acyl-deficient Wg on S239 shows normal secretion, but impaired signalling activity (Komekado et al 2007). In addition, the binding of XWnt8 to its receptor depends on palmitoleic acid addition on Ser187 (Janda et al 2012). Furthermore, the activity of the XWnt5a and XWnt11 (Xenopus Wnt homologues) is regulated by tyrosine sulfation that promotes their interaction, resulting in the formation of Wnt11/5a complexes with enhanced signalling activity (Cha et al 2009). These studies highlight the necessity in exploring further the biosynthesis of Wnt proteins, as Wnt secretion and function is highly regulated at the post-translational level.

### 1.6.2 Wnt secretion

The mechanisms that regulate the secretion of Wnt factors are not well understood and the only evidence that we have are based on studies in the *Drosophila* neuromuscular junction (NMJ) and the Wnt homolog Wg. After Wg exits the ER interacts with Evenness interrupted, also known as Wntless, (Evi/Wls), a transmembrane protein present in the Golgi, endocytic vesicles and

the plasma membrane (Banziger et al 2006, Bartscherer et al 2006). Evi promotes Wg release, whereas loss of function of Evi leads to accumulation Wg in the producing cells (Banziger et al 2006, Bartscherer et al 2006, Korkut et al 2009), demonstrating that Evi is required for the transport of Wg to the cell membrane. Moreover, *evi* mutant flies have patterning defects, mimicking the *wingless* mutants (Bartscherer et al 2006). Interestingly, this is an evolutionary conserved mechanism as the *C. elegans* Evi/Wls homolog is required for proper spindle-orientation during early development, similar to the Wnt homolog MOM-2 (Banziger et al 2006). However, the mechanisms that regulate the secretion of mammalian Wnts has not been described.

At the synapse level, Evi/Wls promotes Wg release from presynaptic boutons into the synaptic cleft of the *Drosophila* NMJ via exosome-like vesicles (Korkut et al., 2009). Importantly, loss of function of Evi/Wls leads to Wg accumulation in the Golgi (Korkut et al 2009). Interestingly, Evi/Wls is not only involved in Wg secretion, but it is transported across synapses and recruits dGRIP, a Wg-receptor-interacting protein important for Wnt-mediated signal transduction, to postsynaptic sites (Korkut et al 2009). It remains to be determined whether this mechanism is conserved to other type of synapses. Another more recent study showed that Wg is also secreted in the NMJ by subperineurial glial cells, resulting in glutamate receptor clustering (Kerr et al 2014). However, whether Evi/Wls is also involved in this process is not known yet.

### 1.6.3 Wnt signalling pathways

During the last two decades great progress has been made in elucidating the signalling pathways activated by Wnt proteins (Figure 1.5), broadly categorized as canonical and non-canonical pathways (Behrens 2013, Gordon & Nusse 2006, Kohn & Moon 2005, Niehrs 2012, Salinas 2007, van Amerongen & Nusse 2009, Veeman et al 2003). The best understood cascade is the ß-catenin (or canonical) pathway where binding of Wnts to their Frizzled receptors (Fz) and their co-receptors LRP5/6 activates a signalling cascade resulting in changes in gene expression. Downstream of these receptors is Dishevelled (Dvl), a scaffold protein, which is required for all Wnt signalling cascades. There are three Dvl genes in mammals, each of these having both unique and redundant functions, as revealed by mutant analysis (Etheridge et al 2008, Wynshaw-Boris 2012).

Activation of Dvl leads to the inhibition of the Glycogen synthase kinase 3Β (Gsk3Β), a serine/threonine kinase, and the subsequent accumulation of the cytoplasmic protein ß-catenin which translocates to the nucleus to promote TCF/LEF-mediated transcription (Gordon & Nusse 2006). In addition, a divergent canonical pathway, which does not signal to the nucleus but still requires Gsk3Β inhibition, regulates cytoskeletal changes (Salinas 2007). The next well-known Wnt cascade is the planar cell polarity pathway (PCP), which through Fz receptors and Dvl leads to the activation of small Rho-GTPases to control cell and tissue polarity (Veeman et al 2003). The third pathway is the calcium-signalling cascade, which results in elevation of intracellular calcium and activation of calcium/calmodulin dependent protein kinase II (CaMKII) (Kohn & Moon 2005). In addition to the Fz and LRP5/6 receptors, the tyrosine kinase receptors Derailed/Ryk and Ror1/2 also activate, or co-activate together with Frizzled receptors, both Wnt canonical and non-canonical pathways (Niehrs 2012). At the *Drosophila* NMJ, an additional pathway has been described (Figure 1.6) (Mathew et al 2005). This pathway is activated by the binding of Wg, which is released from the presynaptic motor neuron, to the Frizzled 2 (Dfz2) receptor on the postsynaptic muscle cell. Upon Wg binding, the C-terminus of the DFz2 receptor is cleaved and transported to the nucleus (Mathew et al 2005). Thus, Wnts can activate different pathways and elicit a variety on responses depending on the cellular context.

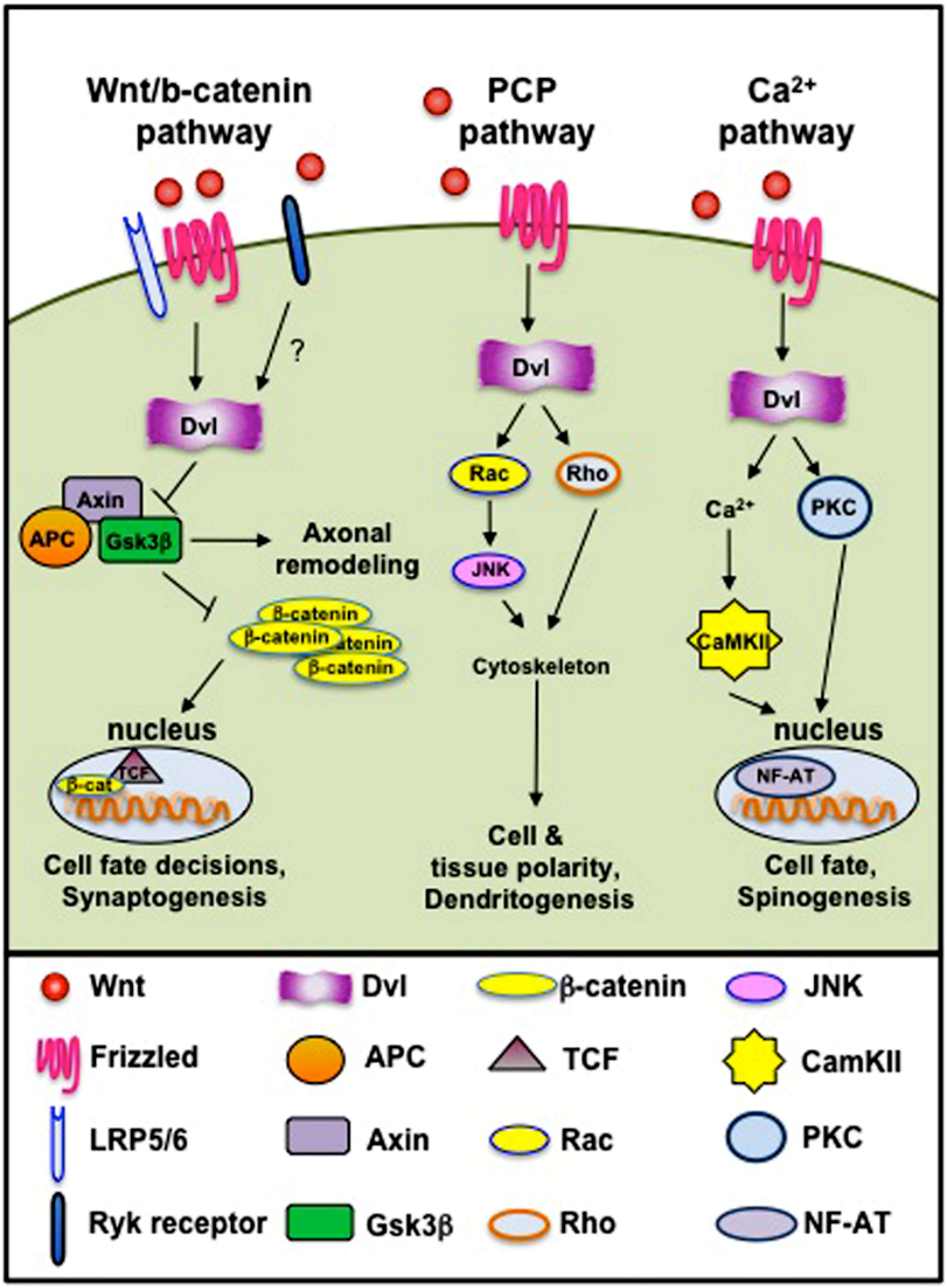



**Figure 1.5: Wnt signalling pathways.** In the β-catenin (or canonical) pathway binding of Wnts to Frizzled (Fz) and LRP5/6 receptors activates Dishevelled (Dvl), which then inhibits Gsk3β, leading to β-catenin accumulation and activation of TCF-mediated transcription. A divergent canonical pathway, which requires Gsk3β inhibition but is transcription-independent, induces axonal remodelling. In the PCP pathway Wnts through Fz receptors and Dvl regulate cell polarity and dendritogenesis through the activation of small Rho-GTPases. The calcium-signalling cascade leads to the activation of CaMKII and PKC to regulate cell fate decisions and spinogenesis.

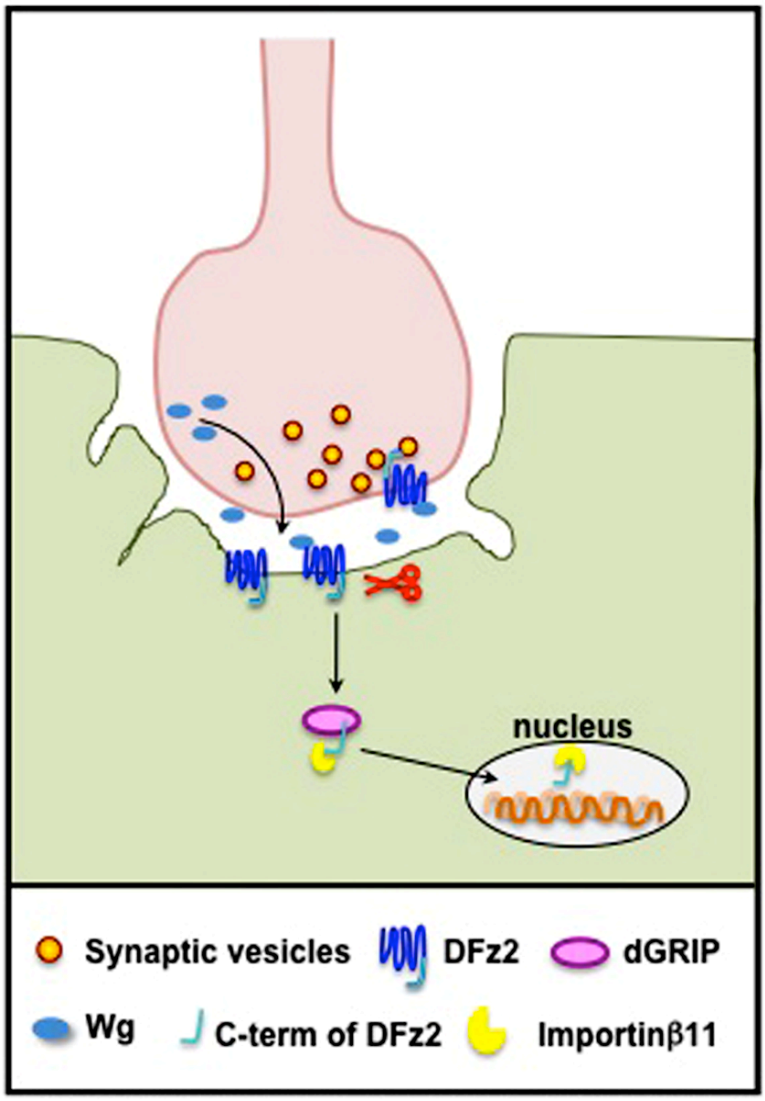



**Figure 1.6: At the *Drosophila* NMJ, Wg activates a pathway that requires the processing of the Wnt receptor DFz2.** Wg, secreted from presynaptic boutons, binds to DFz2 present in both sides of the synapse. In muscle cells, Wg binding to DFz2 triggers the cleavage and nuclear translocation of the C-terminus tail of this receptor. dGRIP and importinβ11 are required for the trafficking of the cleaved tail of DFz2 to the nucleus.

### 1.6.4 Wnt signalling in synapse formation

Expression of Wnts, their receptors and signalling components in the nervous system during the formation of neuronal connections suggested that Wnt signalling plays a role in different aspects of neuronal circuit assembly and function in the adult. The role of Wnt signalling in synapse formation was first demonstrated in cultured neurons (Lucas & Salinas 1997). Wnt7a, which is released from cerebellar granule cells, stimulates the presynaptic differentiation of mossy fiber terminals as demonstrated by the accumulation of the presynaptic

marker Synapsin I (Hall et al 2000). Importantly, loss of function of Wnt7a results in defects at the accumulation of presynaptic components at mossy fibre terminals, which make synapses with granule cell dendrites. These results suggest that Wnt7a acts as a retrograde signal from granule cells to mossy fibre axons (Hall et al 2000). Since then, great progress has been made in elucidating the role of Wnts in synaptogenesis and dissecting the signalling pathways activated at the synapse. Wnt proteins regulate synaptogenesis in both vertebrates and invertebrates by acting as pro- or anti-synaptogenic factors (Inestrosa & Arenas 2010, Koles & Budnik 2012, Park & Shen 2012, Sahores & Salinas 2011).

Although initial studies focused on the role of Wnts on axonal remodelling and presynaptic assembly, Wnts can signal to the postsynaptic side to promote postsynaptic development and synaptic strength (Ciani et al 2011, Henriquez et al 2008, Jensen et al 2012, Mathew et al 2005). Here I discuss the recent advances in understanding the role of different Wnt signalling pathways in synaptic assembly.

### 1.6.5 Wnts on axonal remodelling

During early stages of synapse formation, Wnts act as target-derived signals to induce terminal axonal remodeling, a process characterized by axonal spreading, growth cone enlargement and branching. This remodeling precedes presynaptic assembly (Hall et al 2000, Krylova et al 2002, Lucas & Salinas 1997, Purro et al 2008). In the cerebellum, Wnt7a is released from granule cells and acts on incoming mossy fiber axons to induce axonal remodeling and Synapsin1 clustering (Hall et al 2000). Importantly, Wnt7a signalling is required for axonal remodeling *in vivo*, since *Wnt7a* mutant mice show defects in mossy fibre axonal remodelling and in the accumulation of synaptic proteins (Hall et al 2000). Gain of function of Dvl1 mimics Wnt7a effects by increasing the clustering of synaptic proteins. Conversely, *Dvl1* mutant mice exhibit significantly fewer synaptic clusters (Ahmad-Annuar et al 2006). Moreover, *Wnt7a*/*Dvl1* double mutant mice exhibit a more severe phenotype than single mutants (Ahmad-Annuar et al., 2006). Consistent with the role of the canonical Wnt pathway in this process, inhibition of Gsk3β mimics the effects of Wnt7a on axonal remodelling and Synapsin1 clustering (Hall et al 2002, Hall et al 2000). These studies demonstrate

that Wnt7a signals through Dvl1 and Gsk3β to stimulate axonal remodeling in the cerebellum. In the fly neuromuscular junction (NMJ), Wg, another member of the Wnt family, is required for the proper formation of synaptic boutons through Shaggy/Gsk3 inhibition and activation of the divergent canonical Wnt pathway (Franco et al 2004, Miech et al 2008, Packard et al 2002). In the spinal cord, Wnt3 expressed by motor neurons promotes the remodelling of NT-3 responsive dorsal root ganglia neurons (Krylova et al 2002) that make direct synaptic connections to motor-neuron dendrites in the ventral spinal cord. Axon remodelling is characterized by growth cone enlargement, increased growth cone complexity and axon branching. Therefore, Wnt proteins are target-derived signals that induce extensive structural remodelling of presynaptic axonal terminals.

During axon remodelling, Wnts induce profound changes in the organisation of microtubules (MTs). Time-lapse recordings from EB3-GFP expressing cells revealed that Wnt3a induces the loss of microtubule directionality, resulting in microtubule looping and growth cone pausing (Purro et al 2008). Wnt3a acts through a divergent canonical β-catenin pathway that is independent of transcription, but requires Dvl1 and Gsk3β inhibition to induce loss of the microtubule plus-end binding protein Adenomatous polyposis coli (APC) from the MT plus-ends (Figure 1.7), resulting in defects in the directionality of MT growth (Purro et al 2008). Similarly, studies at the *Drosophila* NMJ revealed that the divergent canonical Wnt pathway promotes axonal remodelling and MT looping (Franco et al 2004, Miech et al 2008). Consistently, *wg* mutants have defects in synaptic bouton formation and morphology (Miech et al 2008, Packard et al 2002). Together, these studies demonstrate that Wnt signalling factors target the microtubule cytoskeleton to drive axons to their synaptic targets.

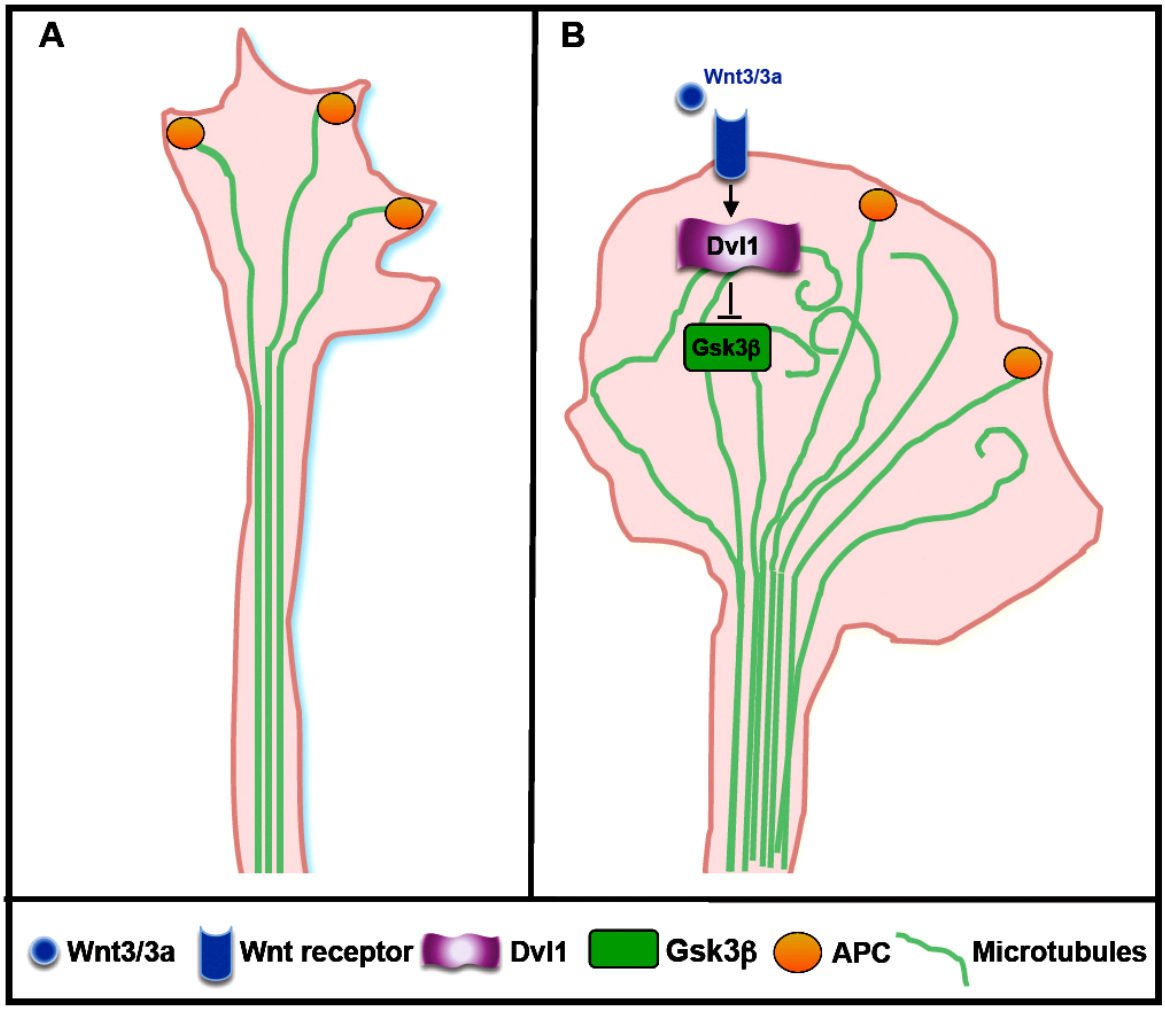



**Figure 1.7: Wnt3/3a promotes axonal remodelling in DRG neurons.** (A) The microtubule plus-end protein APC tethers microtubules to the leading edge of growth cones. (B) Wnt3/3a induces the loss of APC from microtubule ends resulting in microtubule looping and growth cone enlargement.

### 1.6.6 Wnts on presynaptic assembly

Wnt signalling factors are also important for the recruitment of presynaptic components. Wnt7a through the Fz5 receptor, Wnt5a through the Ror1/2 receptors and Wnt3a through Fz1 promote the clustering of presynaptic proteins (Paganoni et al 2010, Sahores et al 2010, Varela-Nallar et al 2009). Importantly, blockade of canonical Wnt signalling using the specific endogenous inhibitor Dickkopf 1 (Dkk1) or inhibition of Gsk3ß mimic Wnt-induced presynaptic clustering (Ahmad-Annuar et al 2006, Davis et al 2008, Hall et al 2000, Lucas & Salinas 1997), suggesting a role for the canonical Wnt signalling pathway in presynaptic assembly. However, inhibition of transcription does not affect Wnt7a-mediated presynaptic assembly (Dickins and Salinas, unpublished data), suggesting that Wnt7a might act locally to regulate Dvl1 and Gsk3ß and promote presynaptic assembly by recruiting synaptic components (Figure 1.8). Indeed,

presynaptic clustering induced by Wnt7a, Wnt5a and Wnt3a is not associated with a change in total protein levels (Ahmad-Annuar et al 2006, Cerpa et al 2008, Ciani et al 2011, Varela-Nallar et al 2009). Together these findings indicate that Wnts promote presynaptic assembly by promoting the recruitment of existing synaptic proteins and SVs via a divergent canonical pathway.

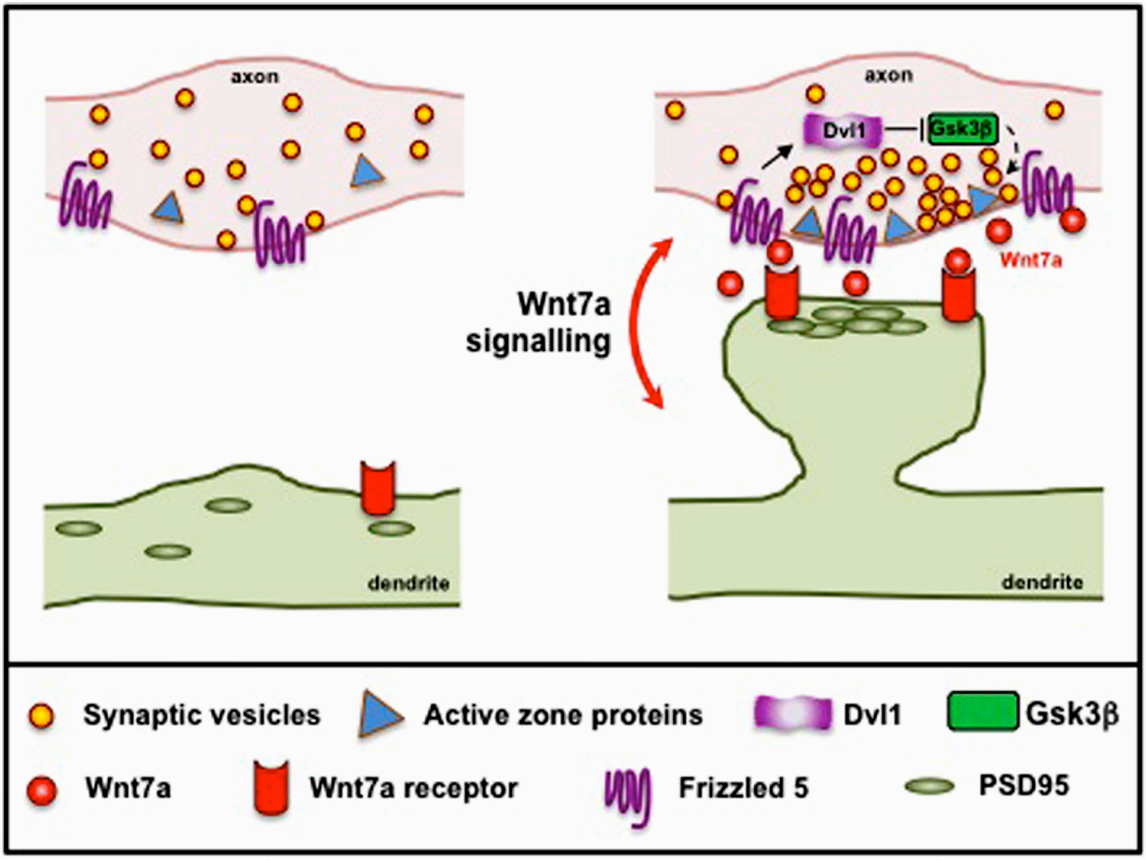



**Figure 1.8: Wnt7a acts bi-directionally to promote synaptic assembly.** On axons, Wnt7a binds to Fz5 receptors and induces the recruitment of presynaptic components, including synaptic vesicles and active zone proteins. On dendrites, Wnt7a promotes PSD95 clustering and spine formation and growth, through an unidentified receptor.

## 1.6.7 Wnts on postsynaptic assembly

### Excitatory synapses

Four different members of the Wnt family have been shown to induce postsynaptic assembly and spine formation: Wnt7a, Wnt5a, Wnt2 and more recently Wnt8a (Ciani et al 2011, Farias et al 2009, Hiester et al 2013, Sharma et al 2013, Varela-Nallar et al 2010). However, since Wnts play a crucial role in presynaptic assembly at central synapses (Ahmad-Annuar et al 2006, Hall et al 2000, Lucas & Salinas 1997, Paganoni et al 2010, Sahores et al 2010, Varela-

Nallar et al 2009), this raises the question as to whether the effect on the postsynaptic side is indirect following the assembly of the presynaptic bouton.

A direct role of Wnt signalling in the postsynaptic differentiation of excitatory synapses was demonstrated by gain and loss of function studies of Dvl1, a scaffold protein and a key component of the Wnt pathway (Ciani et al 2011). While gain of function of Dvl1 mimics the effect of Wnt7a on spine morphogenesis, loss of function studies provided evidence that Dvl1 is required on dendrites for Wnt7a to regulate spine formation (Ciani et al 2011). These findings demonstrate that Wnt signalling is important for the coordinated development of both sides of the synapse (Figure 1.8).

Consistent with the effect of Wnt7a signalling on excitatory synapse formation, spine formation and growth, Wnt7a increases both the frequency and the amplitude of miniature excitatory postsynaptic currents (mEPSCs) (Ciani et al 2011). Importantly, double mutant mice for *Wnt7a* and *Dvl1* exhibit strong deficits in spine morphogenesis and synaptic strength in the hippocampus (Ciani et al 2011). Postsynaptic Wnt7a signalling promotes PSD95 clustering and the rapid local activation of CaMKII within dendritic spines (Figure 1.9A) (Ciani et al 2011). Notably, CaMKII is required for Wnt7a-mediated spine growth and increased synaptic strength. These findings clearly demonstrate a novel role for Wnt7a signalling in promoting postsynaptic differentiation and maturation through CaMKII, a key molecule involved in synaptic plasticity (Coultrap & Bayer 2012, Lisman et al 2012).

Studies on Wnt5a, another Wnt family member, suggest its role in excitatory synapse formation, but these findings are controversial. Wnt5a induces postsynaptic differentiation at excitatory synapses in hippocampal neurons by rapidly inducing the clustering of PSD95 through a non-canonical pathway that requires JNK activation (Figure 1.9A) (Farias et al 2009). Although it has been reported that Wnt5a promotes spine morphogenesis through a calcium-dependent signalling pathway (Varela-Nallar et al 2010), other studies indicate that Wnt5a does not affect spine formation (Cerpa et al 2011, Farias et al 2009). Further analyses including *in vivo* loss of function studies might resolve these apparent conflicting results. Intriguingly, it has been shown that short

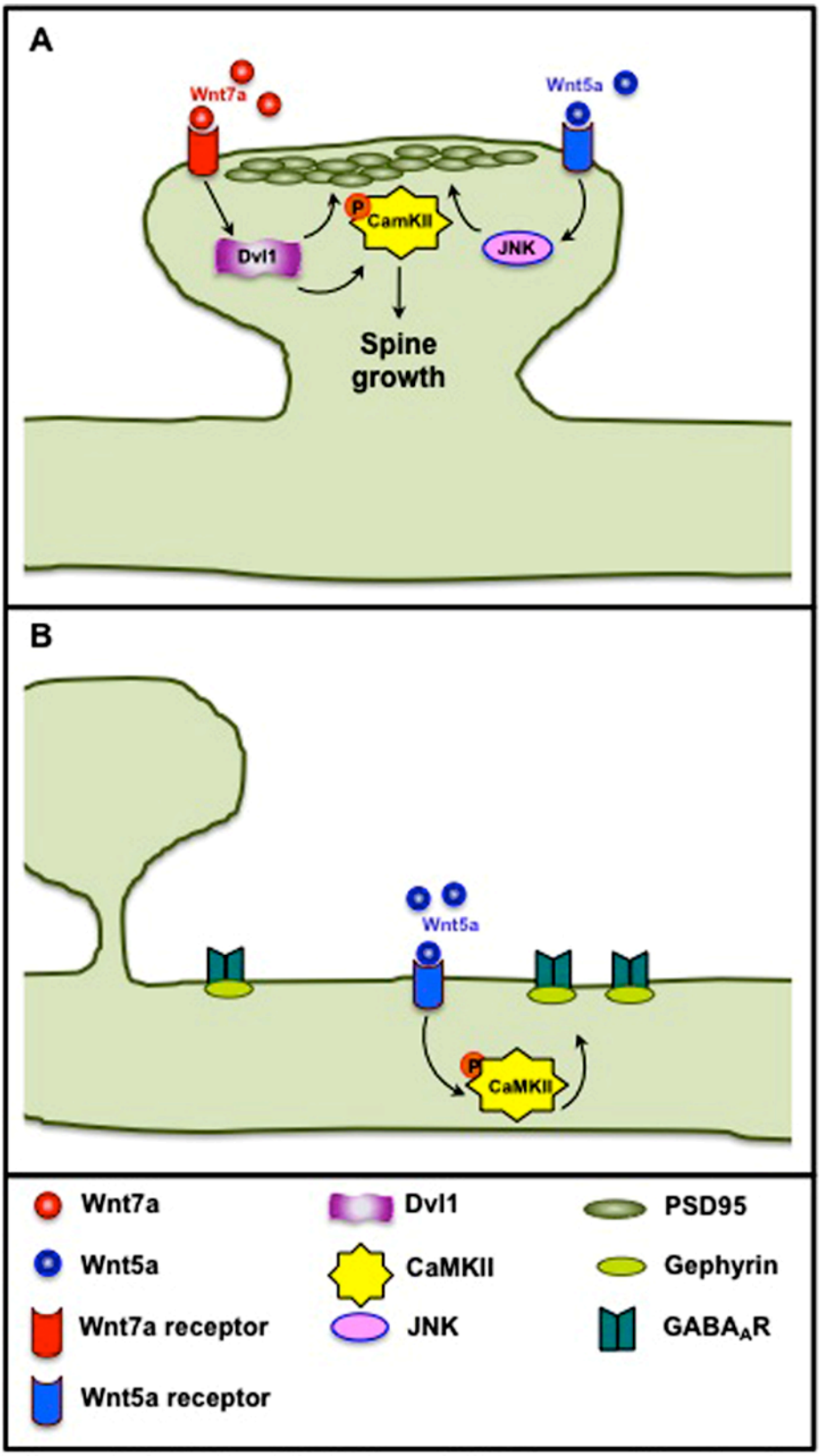



**Figure 1.9: Wnt5a and Wnt7a regulate the assembly of central synapses.** Wnt7a and Wnt5a induce the formation of postsynaptic structures at excitatory synapses (A). Wnt7a, through Dvl1 and CaMKII, induces the recruitment of PSD95 and increases spine growth. In contrast, Wnt5a induces PSD95 clustering via JNK. (B) Wnt5a also promotes the formation of inhibitory synapses by inducing the clustering of $GABA_A$ receptors through CaMKII at inhibitory synapses.

exposure to Wnt5a does not affect presynaptic assembly, whereas other studies have reported that Wnt5a promotes or even inhibits the clustering of presynaptic components (Cerpa et al 2011, Cerpa et al 2008, Davis et al 2008, Farias et al 2009, Paganoni et al 2010, Varela-Nallar et al 2012). Moreover, the frequency of mEPSCs is unaffected by Wnt5a (Cerpa et al 2011). Therefore, it is currently unclear whether Wnt5a is a synaptogenic factor or a postsynaptic modulator.

Recent studies on the role of another Wnt family member, Wnt8a, revealed that the canonical Wnt pathway is also directly involved in postsynaptic formation (Sharma et al 2013). Wnt8A promotes excitatory synapse formation, without affecting inhibitory synapses, through the canonical Wnt co-receptor LRP6 (Sharma et al 2013). Importantly, authors showed that postsynaptic expression of LRP6 is required for spine formation both in culture neurons and *in vivo* (Sharma et al 2013). Interestingly, authors showed that LRP6 is exclusively localized in excitatory synapses (Sharma et al 2013), supporting the notion that the mechanism that determines the type of synapses that will be form upon Wnt signalling activation is the localization of the Wnt receptor. However, another more recent study showed that *in vivo* loss of LRP6 at the postnatal stage results in late onset impairment (18 months and onwards) in synaptic maintenance and plasticity, without any obvious defects in spine formation at 3 or even 6 months of age (Liu et al 2014). Therefore, the role of the LRP6 receptor and the canonical Wnt pathway is postsynaptic development is controversial.

Very recently, the role of Ror2, another Wnt receptor, has also been reported (Alfaro et al 2015, Cerpa et al 2015). The tyrosine kinase receptor Ror2, which is localised in close proximity to both pre- and postsynaptic markers as well as in spine heads, is required for spine morphogenesis *in vitro* (Alfaro et al 2015). Interestingly, ROR2 overexpression increases spine growth without affecting spine density (Alfaro et al 2015), mimicking the effect induced by gain of function of Dvl1 (Ciani et al 2011). Importantly, Ror2 signalling through PKC and JNK increases NMDAR-mediated synaptic transmission (Cerpa et al 2015). However, the Wnt ligands that bind Ror2 and its role in spine formation *in vivo* remain undetermined.

In cortical neurons, exposure to the Wnt antagonist Sfrp1 results in fewer and smaller spines, suggesting a role for endogenous Wnts in spine morphogenesis in

the cortex (Hiester et al 2013). Indeed, Wnt2, which is expressed in several cortical areas, promotes spine morphogenesis (Hiester et al 2013). Although the pathway activated by Wnt2 remains to be determined, studies on Wnt7a, Wnt8a and Wnt5a demonstrate that different signalling pathways promote postsynaptic assembly.

### Inhibitory synapses

Synapses containing γ-aminobutyric acid (GABA) receptors represent the main inhibitory synapses in the central nervous system. Inhibitory synapses are located on the cell body and at the shaft of proximal dendritic branches. At these synapses, Gephyrin, the main scaffolding protein, regulates the clustering of $GABA_A$ receptors (Fritschy et al 2008, Fritschy et al 2012, Jacob et al 2005). Although several signalling molecules have been shown to promote the assembly of inhibitory synapses (Lu et al 2009, Luscher et al 2011, Takayama 2005), the molecular mechanism remains poorly understood.

In addition to its role in excitatory synapses, Wnt5a modulates inhibitory synapses by increasing the surface localisation and clustering of $GABA_A$Rs without affecting the total levels of these receptors (Cuitino et al 2010). This is accompanied by an increase in the amplitude, but not frequency, of miniature inhibitory postsynaptic currents (mIPSCs) and increased evoked inhibitory postsynaptic currents (eIPSCs). This effect induced by Wnt5a is mediated by CaMK signalling (Figure 1.9B) (Cuitino et al 2010). Intriguingly, Wnt5a does not affect the clustering of Gephyrin despite affecting the clustering of $GABA_A$Rs. Together these findings suggest that Wnt5a promotes the localization of GABA receptors on existing synapses, therefore increases the amplitude of mIPSC, without affecting the number of inhibitory synapses.

In sharp contrast with Wnt5a, Wnt7a and Wnt8a have no effect on inhibitory synapses. Gain of function on cultured neurons demonstrates that Wnt7a does not affect the formation of inhibitory synapses nor does affect GABA-mediated mIPSCs (Ciani et al 2011). Similarly, Wnt8a does not induce the clustering of inhibitory synaptic markers (Sharma et al 2013). Therefore, Wnt7a and Wnt8a specifically promote the formation of excitatory synapses, thus affecting the ratio of excitatory/inhibitory inputs. These results suggest that Wnt7a and

Wnt8a signalling could serve as targets for the treatment of neurological disorders where an imbalance between excitatory and inhibitory synapses is observed, such as autism and epilepsy.

In summary, distinct Wnt signalling pathways contribute to the formation of different types of synapses. Interestingly, activation of the same signalling pathway can lead to the formation of different synapse types. For example, Wnt7a signalling activates CaMKII to promote spine growth, whereas Wnt5a also through CaMKII increases the clustering of synaptic components at inhibitory synapses. These findings suggest that different receptor complexes might contribute to these very distinct outcomes. Future studies on the localization and activation of Wnt receptors will shed new light into the specific molecular events that lead to the assembly of postsynaptic structures at different types of synapses.

### 1.6.8 Wnts on synaptic plasticity

Several studies have implicated Wnt signalling in activity-mediated synapse formation. First, neuronal activity induces the expression and/or release of Wnts (Chen et al 2006, Gogolla et al 2009, Wayman et al 2006, Yu & Malenka 2003). Wnt2 and Wnt3a expression and release, respectively, are elevated upon NMDAR activation through KCL depolarisation or titanic stimulation (Chen et al 2006, Wayman et al 2006). In addition, Wnt7a/b protein levels are increased by KCL depolarization *in vitro* (Tabatadze et al 2014) and in response to environmental enrichment (EE) or to spatial learning *in vivo* (Gogolla et al 2009, Tabatadze et al 2012). Also, Wnt3a or Wnt5a facilitates LTP expression in the hippocampus (Cerpa et al 2011, Chen et al 2006). Finally, blockade of endogenous Wnt signalling using the canonical antagonist Dkk1 or the broad Wnt scavenger Sfrp1 impairs LTP (Cerpa et al 2011, Chen et al 2006). Together these studies raise the exciting possibility that Wnt factors are important modulators in activity-dependent responses.

In addition, to Wnt factors the surface and synaptic levels of the Frizzled-5 (Fz5) receptor are also regulated by neuronal activity (Sahores et al 2010). In particular, high-frequency stimulation (HFS) promotes the recruitment of surface Fz5 (sFz5) in synapses without affecting its total proteins levels. In contrast,

low-frequency stimulation (LFS) has the opposite effect. Importantly, Wnt-Fz5 signalling is required for both sFz5 localisation on synapses and activity-dependent synapse formation, since Wnt blockade with the extracellular domain of Fz5 (Fz5-CRD) completely abolishes the effect of neuronal activity on sFz5 synapse recruitment and synapse formation (Sahores et al 2010). Thus, induced neuronal activity elicited by HFS regulates the release and/or expression of Wnt factors that bind to Fz5 and promote its synaptic localisation and subsequently synapse formation. These results suggest the exciting possibility that Wnts play a central role in experience-dependent synapse formation including synaptic changes associated with learning and memory.

## 1.7 Thesis Aims

Wnt proteins are well known secreted signalling molecules that regulate different processes that contribute to the formation of neuronal connections from axonal and dendritic outgrowth, as well as synapse formation and elimination (Budnik & Salinas 2011, Dickins & Salinas 2013, Okerlund & Cheyette 2011, Oliva et al 2013, Park & Shen 2012, Purro et al 2014, Sahores & Salinas 2011, Salinas 2012, Varela-Nallar & Inestrosa 2013). Although, during the last decade great progress has been made in discovering the signalling pathways that regulate these events, we have little understanding of how Wnts regulate axonal remodelling and postsynaptic assembly. Previous studies from our lab have demonstrated that Wnt7a released by granule cell neurons in the cerebellum acts on incoming mossy fibre axons to induce growth cone enlargement and axonal spreading, processes that accompanied the assembly of the synaptic bouton (Hall et al 2000). Importantly, Wnt7a deficient mice exhibit defects in axonal terminal remodelling and the accumulation of synaptic proteins at mossy fibre axons (Ahmad-Annuar et al 2006, Hall et al 2000). In the spinal cord, motor neuron-derived Wnt3 promotes the axonal terminal remodelling of NT-3 responsive dorsal root ganglia (DRG) neurons (Krylova et al 2002, Purro et al 2008). This remodelling is manifested by axonal pausing, growth cone enlargement and microtubule looping (Krylova et al 2002, Purro et al 2008). Detailed examination of the mechanisms by which Wnts induce axonal remodelling revealed that microtubule looping is due to the loss of microtubule directionality (Purro et al 2008). However, the effects of Wnts on axonal morphology, such as axonal spreading and growth cone enlargement, suggest

that Wnts could also modulate the actin cytoskeleton, but the molecular mechanisms involved remain elusive.

Wnt7a promotes dendritic spine formation and growth in the hippocampus. Postsynaptic activation of the Wnt signalling pathway, by expressing Dishevelled 1 (Dvl1), partially mimics the effect of Wnt7a by increasing spine growth, but not spine number (Ciani et al 2011). However, it is unclear how Wnt signalling modulates the actin cytoskeleton to induce spine formation and growth. The main aim of this thesis was to define the pathway(s) by which Wnt signalling regulates axonal remodelling and postsynaptic differentiation through changes in the actin cytoskeleton. In particular I focused my work on four specific aims:

I. To identify new modulators of Wnt signalling in neurons

II. To test the function of Eps8, an interactor of Dvl1 identified in Aim I, in Wnt-mediated axonal remodelling

III. To examine the role of Eps8 in Wnt-mediated postsynaptic formation

IV. To examine the role of Eps8 in activity-mediated spine formation

# Chapter 2:

# Materials & Methods

# 2.1 Cell cultures

## 2.1.1 Hippocampal neuronal cell cultures

Primary neurons were prepared from E18 embryos of Sprague-Dawley rats killed by cervical dislocation according to (Dotti et al 1988) and following Home Office regulations. In brief, the hippocampi were dissected in ice-cold Hank's balanced salt solution (HBSS). Once dissected, the hippocampi were incubated in 0.25% trypsin (Invitrogen; cat. No:15090046) for 18 minutes at 37°C in HBSS. After the 18 mins incubation trypsin was washed 3 times with HBSS. Finally, the hippocampi were triturated using three glass Pasteur pipettes that were flame-polished to achieve 3 different pore sizes. Cells were counted with a Neubauer haemocytometer and then plated on 12mm glass coverslips (VWR; cat. No: 1001/12) pre-treated with nitric acid overnight, sterilised for 6 hours at 200°C and then coated with poly-L-lysine (1 μg/mL in borate buffer). Cells were initially plated in MEM10 medium (DMEM containing Glutamax and supplemented with 10%v/v Horse serum (Invitrogen; cat. No: 16050130), 1 mM sodium pyruvate and 0.2% v/v Pen/Strep) and 2 hrs later MEM10 was replaced with Neurobasal medium supplemented with N2 (Invitrogen), B27 (Invitrogen), 25 mM D-glucose and 1 mM L-glutamine. Neurons were plated at 250 cells/$mm^2$ for either calcium transfection or they were transfected with Amaxa and then plated at 100 cells/$mm^2$. All cultures were maintained at 37°C/5% CO2.

## 2.1.2 Dorsal root ganglia (DRG) neuronal cell culture

DRG neurons were isolated from newborn rats (P0-P3) or E18 rat embryos. DRGs were dissected in ice-cold Hank's balanced salt solution (HBSS). Once dissected, they were centrifuged for 5 mins at 110g and then incubated with 5 mg/mL dispase (Invitrogen; cat. No: 1710504) in HBSS for 30 mins at 37°C. Dispase was then diluted 10 times with HBSS and the DRGs were centrifuged again for 5mins at 110g. Finally, DRGs were resuspended in seeding medium (DMEM with Glutamax, 5% v/v Horse serum, 5% v/v Fetal Bovine Serum, 50 ng/mL NGF and 0.2% v/v Pen/Strep) and were triturated using a P1000 tip and a extra narrow flamed-polished glass Pasteur pipette. Neurons were plated at 100 cells/$mm^2$ on glass coverslips (VWR; cat. No: 1001/12) treated with nitric acid overnight, sterilised overnight at 200°C, coated with poly-L-lysine (1 μg/mL in borate

buffer) for 3 hours and finally with laminin (20 mg/mL) for 3 hours. Two hours later seeding medium was replaced to Neurobasal medium supplemented with N2 (Invitrogen), B27 (Invitrogen), 25 mM D-glucose, 1 mM L-glutamine, NGF (50 mg/mL) and 0.2% v/v Pen/Strep.

### 2.1.3 NB2a cell cultures and differentiation

NB2a cells were cultured in DMEM medium with Glutamax supplemented with 10% FBS and 1% v/v Pen/Strep. For immunofluorescence analyses cells were plated on 13 mm glass coverslips (VWR; cat. No: 6310149). To differentiate them and extend neurites, NB2a cells where maintained overnight in the absence of serum in Optimem medium supplemented with Glutamax and 1 μM dibutyryl-cyclic-AMP (Sigma).

### 2.1.4 Rat1B cells and Wnt3 conditioned media

For experiments where DRG neurons were treated with Wnt3a, mouse recombinant Wnt3a was purchased from R&D Systems and used at a concentration of 50 ng/mL. However, for some experiments Wnt3-conditioned media was produced from Rat1B cells stably transfected with a pLNCX retroviral construct expressing Wnt3-HA. Rat1B cells were cultured in DMEM medium supplemented with Glutamax, 10% FBS, and pen/strep. Cells expressing Wnt3-HA were selected with 250 mg/mL G418. Neurobasal medium supplemented with N2 (Invitrogen), B27 (Invitrogen), 25mM D-glucose, 1mM L-glutamine and pen/strep was added on the cells when they had reached approximately 80% confluency and was conditioned for at least 16 hours. The conditioned medium was then collected and centrifuged at 220 g for 5 mins to remove any debris or detached cells. Before application to DRG neurons, conditioned medium was diluted with fresh cultured medium (1:1) and NGF was added at a final concentration of 50 ng/mL. G418-resistant Rat1B cells were used as control. The level of Wnt3-HA protein was determined by Western blot using an anti-HA antibody (Roche).

# 2.2 Transfection methods

## 2.2.1 Calcium phosphate

Hippocampal cultures were transfected at 7-8 DIV with EGFP-actin, Eps8-myc, scramble shRNA, 3 specific shRNAs against Eps8, Eps8Δ1-585, Eps8Δ180-585, Eps8Δ533-821 (Eps8ΔC), Eps8Δ550-693 and Dvl1-HA constructs by the calcium phosphate method (Dudek et al 2001). Briefly, the culture medium was removed from the culture dish, kept at 37°C/5% CO2 and replaced with transfection medium (Neurobasal medium supplemented with 35mM D-glucose). For each p60 dish (21cm$^2$), 6μg of total DNA was precipitated for 25 minutes with calcium (final concentration 125 mM) in Hepes-buffer solution (HBS; 42mM Hepes, 274 mM NaCl, 10 mM KCl, 1.4 mM $Na_2HPO_4.7H_2O$, 15 mM D-glucose, pH 7.06-7.14) in the dark and was then applied onto the cells for 10 mins. Cells were then washed three times with transfection medium and finally the old culture medium supplemented with 0.1% v/v Pen/Strep was added to the dish. Co-transfection protocols with EGFP-actin were standardized to achieve the maximum co-transfection efficiency (almost 100%) using a ratio of plasmid DNA 1:3.

## 2.2.2 Lipofectamine

For NB2a transfection, cells were plated the previous day at 400 cells/mm$^2$. For a p60 dish (21cm$^2$), 6 μg of DNA was mixed with 200 μL of Optimem medium. In parallel, 10 μL of Lipofectamine 2000 was mixed with 200 μL of Optimem medium and incubated for 5 mins at room temperature. The Lipofectamine solution was then added drop-wise to the tube containing the DNA and the mix was incubated for 25 mins at room temperature. Meanwhile, the culture media was replaced by 3 mL Optimem medium. The Lipofectamine 2000/DNA mixture was added drop-wise to the dish and the cells were incubated at 37 °C / 5% CO2 for 3 hrs. Finally, the medium was replaced with fresh Optimem medium and 2 hrs after 1 μM dibutyryl-cyclic-AMP (Sigma) was added to induce differentiation.

### 2.2.3 Amaxa nucleofection

For Amaxa electroporation, cells were transfected before platting. For transfection of hippocampal neurons, three million cells were centrifuged for 5 mins at 110 g and then re-suspended in 100 mL of transfection solution for rat primary neurons. 3 μg of EGFP-actin was mixed well with the cell suspension and then the cells were transfected using the program G13, following manufacture's instructions. Immediately after, warm MEM10 was added to the cells to help them recover quickly.

For transfection of rat DRG neurons cells were centrifuged at 110 g for 5 mins and then re-suspended in 100 mL of transfection solution for rat primary neurons. 4-6 μg of total DNA was mixed well with the cell suspension and then neurons were transfected using the program G13. Immediately after, warm seeding medium (DMEM medium supplemented with Glutamax, 5% v/v Horse serum, 5% v/v FBS, 50 ng/mL NGF and pen/strep) was added to help the cells to recover quickly. The seeding medium was replaced two hours after to the usual culture medium. Constructs used were GFP (Clontech) Dvl1-HA, Eps8-myc, scramble shRNA, 3 specific shRNAs against Eps8, and Eps8Δ533-821.

## 2.3 Chemical LTP (cLTP)

LTP was induced in 13-14 DIV hippocampal cultures using a cLTP protocol (200 mM glycine for 10 mins in the absence of $Mg^{2+}$) (Fortin et al 2010, Oh & Derkach 2005). In brief, prior to potentiation, cultures were incubated in warmed control ACSF solution for 30 mins: 125 mM NaCl, 2.5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$ 5 mM Hepes, 33 mM glucose, 0.5 mM TTX, 20 mM AP5, 20 mM bicuculline, and 3 mM strychnine, pH 7.4. cLTP was then induced by treating cultures for 10 mins with glycine in the absence of $Mg^{2+}$, TTX and AP5 (125 mM NaCl, 2.5 mM KCl, 5 mM $CaCl_2$, 5 mM Hepes, 33 mM glucose, 200 mM glycine, 20 mM bicuculline and 3 mM strychnine, pH 7.4) before returning them back to control ACSF solution and incubated for 1 hour at room temperature prior to fixation.

# 2.4 Electrophysiology

AMPA receptor-mediated miniature excitatory postsynaptic currents (mEPSCs) were recorded, using whole-cell patch-clamp configuration, in the presence of 0.1 mM TTX, 10 mM bicuculline and 50 mM AP5, as previously described (Ciani et al 2011). Approximately equal numbers of cells was recorded from scrambled or Eps8 shRNAs-expressing cells on each day from 12-14 DIV cultures. For recordings of cLTP experiments: Krebs extracellular solution was used supplemented with 1 mM $MgCl_2$, 0.5 mM TTX, 20 mM AP5, 20 mM bicuculline and 3 mM strychnine. Analyses were performed using a combination of WinEDR and WinWCP software (freely available at http://spider.science.strath.ac.uk/ sipbs/software ses.htm).

# 2.5 Immunofluorescence, image acquisition and analysis

## 2.5.1 Fixation and immunostaining

Cultures were fixed with 4% PFA/4%sucrose in PBS for 20 mins at room temperature or with 100% ice-cold methanol for 5 minutes at -20°C. Cells were then permeabilized with 0.05% Triton for 5 mins and were then blocked with 5% BSA w/v for 1 hour at room temperature. Antibodies against Eps8 (BD), GFP (Upstate Biotechnology), vGlut1 (Chemicon), PSD95 (Thermo Scientific), GluN1 (Synaptic Systems) and sGluA1 (Calbiochem) were applied in 1% BSA w/v for overnight at 4°C. For surface GluA1, live cultures were incubated with an antibody against the extracellular domain of GluA1 for 15 mins at 37°C prior to fixation. Secondary antibodies conjugated with Alexa 488, Alexa 568, and Alexa 647 (Invitrogen) were applied in 1% BSA w/v for 1 hour at room temperature. Finally, coverslips were mounted on microscope slides 0.8-1 mm using FluorSave reagent (Calbiochem).

## 2.5.2 Image acquisition and analysis of growth cones

Fluorescent images of growth cones from NT3-rensponsive neurons were captured using an Olympus BX60 wide-field microscope with a 100x oil objective (NA= 1.30). At least 50 growth cones were acquired and analysed per condition

using the Metamorph software (Molecular Devices). Growth cone size was determined from the growth cone area, measured manually using the drawing tool. Accumulation of F-actin was determined by setting a high threshold on the Phalloidin images to measure only the red and white area in the pseudocolour images. Identical thresholds were used for control and experimental conditions. % F-actin represents the area of bright F-actin normalised to growth cone area.

### 2.5.3 Image acquisition and analysis of dendritic spines, Eps8 puncta, synaptic puncta

Fluorescence images of neurons with a typical pyramidal morphology were captured using an Olympus FV1000 inverted confocal microscope equipped with a 60x oil objective (NA = 1.40), producing image stacks of 162.7 x 162.7 μm with an average z depth of ≈ 4 μm. 15-25 cells per condition were acquired and analysed. For each cell, 3-4 dendrites were analysed from the maximum projection images using Volocity software (PerkinElmer). Filopodium number, spine number and size were measured manually. Spine size was determined by measuring the width of the spine head. Filopodia were defined as thin protrusions without a distinguishable head, thin spines as thin protrusions with a distinguishable head, stubby spines as short protrusions without a neck, mushroom spines as protrusions with a short neck and a distinguishable head. Spines were classified as irregular when their spine head did not have a typical globulous shape. Synapses were defined by the apposition of pre- and post-synaptic markers, such as vGlut1 and GluN1. For synaptic puncta analysis, thresholding protocols were customised for each experiment based on intensity and size. Protocols were customised to exclude the objects that do not touch the GFP-transfected cell.

### 2.5.4 Fluorescent recovery after photobleaching (FRAP)

FRAP experiments were carried out according to (Koskinen et al 2012) with minor modifications. Image sequences were captured using an Olympus FV1000 inverted confocal microscope with a 60x oil immersion objective (NA = 1.40). FRAP assays were performed at 37°C / 5% CO2 in culture medium. Images were captured using maximum 10% of laser power (488nm) in a 256 x 256 format, 600

Hz speed, 2-line averaging, 2.0 AU (arbitrary unit) pinhole and 3.0 optical zoom. To specify the area of photobleaching, we traced a squared ROI (region of interest) with a maximum area of 7 $\mu m^2$, big enough to cover a single spine. The FRAP protocol was as follows: before bleaching 3 frames with 2 s interval were captured followed by photobleaching using 200 pulses with 100% laser power (maximum bleaching time 6s). Subsequently, 20 frames with 2 s intervals were captured to detect rapid recovery in fluorescence intensity. This was followed by the acquisition of another 20 frames with 5s intervals to avoid laser overexposure of the cells and 10 more frames with 20 s intervals until full recovery of fluorescence was obtained. The total fluorescence intensity of the ROI for each time point was measured using Volocity software (Improvision). The average fluorescence intensity of the first three frames (before photobleaching) was used to normalise the intensity values for each time point. The recovery halftime ($t_{1/2}$) was determined from the average scatter plot, and the first-order rate constant ($k_{obs}$) was calculated using the equation "$k_{obs} = 0.693/t_{1/2}$" (Koskinen et al 2012).

### 2.5.5 Free-barbed end assay

Free-barbed end assays were performed as previously described (Gu et al 2010, Marsick & Letourneau 2011) with minor modifications. In brief, hippocampal neurons were exposed to purified non-muscle rhodamine-conjugated G-actin (Cytoskeleton) that was diluted at 0.45 mM final concentration in saponin permeabilization buffer (20 mM HEPES, 138 mM KCl, 3 mM EGTA, 4 mM MgCl2, 1% w/v BSA, 0.2 mg/mL saponin, 0.5 mM DTT, 1 mM ATP, pH 7.5) for 2 mins. Cultures were immediately fixed with 4% w/v PFA in PHEM buffer (60 mM PIPES, 25 mM HEPES, 10 mM EGTA, 2 mM MgCl2, 0.12 M sucrose (pH 7.0)), treated with 100 mM Glycine in PBS for 10 mins and then stained for GFP (Upstate Biotechnology) and F-actin using Phalloidin conjugated with Alexa647 (Molecular Probes). Images were acquired and analysed as on section 2.5.3. Free-barbed ends were defined as G-actin objects (Rhodamine positive) co-localised with F-actin objects (Phalloidin positive). Following this approach, the false-positive free-barbed ends (that do not co-localise with F-actin) due to insufficiently washed G-actin were excluded.

# 2.6 Biochemistry

## 2.6.1 Isolation of protein extracts from yeast

100 mL of pre-warmed cracking buffer (8 M urea, 5% w/v SDS, 0.4 mg/mL bromophenol blue, 0.1 mM EDTA, 40 mM Tris-HCl, pH 6.8) supplemented with 1% v/v β-mercaptoethanol, 0.1 mg/mL pepstatin A, 0.03 mM leupeptin, 0.37 mg/mL aprotinin and 0.5 mM PMSF and 80 mL glass beads (425–600 µm; Sigma) were added per 7.5 $OD_{600}$ units of yeast pellets transformed with empty pGBDT7 vector or pGBDT7-Dvl1. The samples were heated at 70°C for 10 mins and then vortexed well to break the cells. Lysates where then centrifuged at 13,000 rpm for 5 mins at 4°C and the supernatants were transfer in clean tubes and kept on ice. The tubes containing the pellets were placed at 100 °C for 5 mins and they were vortexed again. The samples were centrifuged at maximum speed for 5 mins at 4°C and the two supernatant fractions were combined. Samples were boiled at 100 °C for 5 mins and immediately run on a SDS/PAGE. Primary antibodies against Dvl1 (Krylova et al 2002) and Myc (Sigma) were used.

## 2.6.2 Immunoprecipitation (IP)

NB2a cells or brain tissue were lysed in Triton buffer (50 mM Hepes, 100 mM NaCl, 4 mM EGTA, 2 mM MgCl2, 0.5% v/v TritonX100, pH 7.4) supplemented with 1 mM PMSF, 1 mM $Na_3VO_4$, 10 mg/mL leupeptin, 10 mg/mL aprotinin, 25 mM NaF and 1 mM pepstatin. Lysates were pre-cleared for 2 hours at 4°C (10 rpm) using G- or A- protein Sepharose beads. The pre-cleared lysates was then incubated overnight at 4°C (10 rpm) with specific an anti-Myc (Sigma) or an anti-Dvl1 (Krylova et al 2002) antibodies or an anti-IgG cocktail (Biorad). The following day G- or A- protein Sepharose beads were added for 2 hrs and subsequently centrifuged and washed 3 times with Triton buffer. The proteins bound to the beads were extracted with equal volume of 2x SDS loading buffer (120 mM Tris, 100 mM DTT, 1.6 g/mL SDS, 0.4 g/mL bromophenol blue, 20% v/v glycerol, pH 6.8). Bead extracts were loaded on SDS/PAGE and antibodies against HA (Roche) or Eps8 (BD) were used.

## 2.6.3 Synaptosomal preparation

Synaptosomes were prepared from adult mouse brains using a sucrose gradient protocol, as previously described (Sahores et al 2010). All the buffers were ice-cold and supplemented with supplemented with 1 mM PMSF, 1 mM $Na_3VO_4$, 10 mg/mL leupeptin, 10 mg/mL aprotinin, 25 mM NaF and 1 mM pepstatin. The samples were kept on ice during the whole procedure. All the centrifugations were performed at 4°C. Brains where homogenized in low sucrose buffer (0.32 M sucrose, 4 mM Hepes pH 7.4) and centrifuged at 800g for 10 mins. The pellet (nuclei) was discarded and the supernatant (S1 -total homogenate) was centrifuged again at 9,000g for 15 min, the supernatant (S2 -cytoplasm) was removed and the pellet (P2 - organelles) was re-suspended in low sucrose buffer. P2 fraction was then placed on top of the sucrose gradient 0.8 M, 1.0 M and 1.2 M sucrose (in 4mM Hepes, pH 7.4) and centrifuged at 65,000g for 45 min. After centrifugation the synaptosomal fraction was isolated (located on 1–1.2M sucrose interface), spun again at 82,500 g for 40 mins and the pellet was re-suspended in low sucrose buffer containing 150 mM NaCl. The synaptosomal fraction was then incubated in rotation at 4°C with an equal volume of 1% Triton X-100 buffer (1% v/v Triton, 0.32 M sucrose, 12 mM Tris, pH 8.0) for 15 min and afterwards was centrifuged at 82,500 g for 45 mins. Finally, the supernatant (S4 - synaptosomal membrane fraction) was removed and the pellet (P4 - postsynaptic density fraction) was re-suspended in low sucrose buffer containing 150 mM NaCl. Protein concentration was estimated by Lowry assay and equal amount of proteins were run onto a SDS/PAGE. Primary antibodies against Eps8 (BD Transduction Laboratories), Syntaxin1 (Developmental Studies Hybridoma bank) and PSD95 (Thermo Scientific) were used. Band intensity was quantified using ImageJ software (National Institutes of Health).

## 2.6.4 Cofillin activity

To measure cofilin activity its phosphorylation on Ser3 was assessed by Western blot. 12-14 DIV hippocampal cultures treated with 100 ng/mL Wnt7a or BSA for 15, 30 or 60 mins were lysed using SDS loading buffer (60mM Tris, 50mM DTT, 0.8 g/mL SDS, 0.2 g/mL bromophenol blue, 10% v/v glycerol, pH 6.8). To determine the downstream pathway that Wnt7a activates to regulate cofilin activity,

cultures were treated with specific inhibitors against Rac1 (100μM; NSC23766) and ROCK (100μM; Y27632) for 30 mins prior to Wnt7a application. Lysates were immediately loaded on SDS/PAGE and specific antibodies against total Cofillin (Abcam) or phospho-Ser3 Cofillin (Abcam) were used. Cofilin activity was determined by the ratio of Phospho/Total cofilin. Band intensity was quantified using ImageJ software (National Institutes of Health).

### 2.6.5 List of antibodies

| Antigen | Species | Supplier | Cat. No | Dilution |
|---|---|---|---|---|
| Myc | rabbit | Sigma | C3956 | 1/2,000 |
| Dvl1 | rabbit | Krylova et al., 2002 | N/A | 1/1,000 |
| HA | rat | Roche | 11867423001 | 1/1,000 |
| Acetylated tubulin | mouse | Sigma | T6793 | 1/3,000 |
| Eps8 | mouse | BD | 610143 | 1/500 |
| GFP | chicken | Upstate Biotechnology | 06-896 | 1/500 |
| Syntaxin | mouse | Developmental Studies Hybridoma bank | 8C3 | 1/3,000 |
| PSD95 | mouse | ThermoScientific | MA1-046 | 1/1,000 |
| vGlut1 | guinea pig | Chemicon | AB5905 | 1/5,000 |
| GluN1 | mouse | Synaptic Systems | 114011 | 1/1,000 |
| Surface GluA1 | rabbit | Millipore | PC246 | 1/100 |
| Cofilin | rabbit | Abcam | ab11062 | 1/500 |
| Prospho-cofilin (Ser3) | rabbit | Abcam | ab12866 | 1/1,000 |

# 2.7 Molecular biology

## 2.7.1 Cloning of yeast two-hybrid clones

Dvl1 full-length cDNA or the PDZ domain of Dvl1 were isolated by PCR using the Dvl1Omega as template and were then cloned into the pGBDT7 vector using the EcoRI and SalI sites. Primers for Dvl1 full length were Fwd: 5'- CGTACAGAATTCGCGGAGACCAAAATCAT -3' and Rvs: 5'- GGCTAGTCGACCATGATG TCCACAAAG -3' and for the PDZ domain Fwd: 5'- AGTACAGAATTCATCA CCGTCACTCTCAACATGGAG -3' and Rvs: 5'- AACTAGTCGACGGCCACTGTGAGA CTGATGGG -3'. Eps8L3 full-length cDNA was isolated by PCR using cDNA prepared from P24 mouse brain and was then cloned onto the pGADT7 vector using the ClaI and XhoI sites. Primers used were Fwd: 5'-AAGCTACTCGAGC TAATGAGTCATCCCCAGCATCCT -3' and Rvs: 5'-AAGTACATCGATATGTCCCGGCCC AGCAGCAGAGCCAT -3'.

## 2.7.2 Plasmid DNA transformation in yeast

AH109 (mat a) or Y187 (mat $\alpha$) yeast strains were transformed using the LiAc method (Yeast protocol Handbook, Clonetech). Yeast cultures were grown in YPDA medium (10 g/L bacto yeast extract, 20 g/L bacto peptone, 20 g/L Glucose monohydrate, 40 mg/L Adenine hemi-sulfate, pH 6.5) for 16 hours at 30 °C (250 rpm). The culture was then diluted 5 times with fresh YPDA medium and was left in incubation for 3 more hours (230 rpm). The $OD_{600}$ after these 3 hours should be around 0.4 to 0.6. If it is not the culture was further diluted or was left to incubate further. The cells were then span down at 1000 g for 5 mins at room temperature, the pellet was washed with distilled water and the cells were span down again at 1000 g for 5 mins at room temperature. Cell pellet was re-suspended in 2 mL of freshly prepared TE/LiAc buffer (10 mM Tris-HCl, 1 mM EDTA, 100 mM LiAc, pH 7.5). Approximately 100 ng of each plasmid was mixed with 100 $\mu$g of boiled salmon sperm DNA (Ambion). The cells were then added to the DNA mixture and were mixed well by vortexing. Five volumes of PEG/TE/LiAc buffer (40% w/v PEG, 10 mM Tris-HCl, 1 mM EDTA, 100 mM LiAc, pH 7.5) was subsequently added and the samples were mixed well by vortexing (~10 secs). The tubes were then incubated at 30 °C for 30 mins (200 rpm). DMSO was

then added (1:10 volume) and the cells were incubated at 42 °C for 15 mins. Immediately after, cells were placed on ice for 1-2 mins and were centrifuged for 5 secs at maximum speed. The pellet was re-suspended in 300 mL of distilled water and transformed cells were plated on 100 mm petri dishes (26.7 g/L minimal SD base, dropout supplement, 20 g/L agar, pH 5.8) lacking either Leucine for the pGADT7 vectors or Tryptophan for the pGBDT7 vectors.

### 2.7.3 cDNA library and bait mating

The AH109 yeast strain (mat a), transformed with pGBDT7-Dvl1, was mated with the Y187 strain (mat $\alpha$), transformed with a cDNA library isolated from adult mouse brain (Clonetech), according to the manufacture's instructions. In brief, AH109 yeast transformed with pGBKT7-Dvl1 (bait) were growth for 24 hours at 30°C in 100 mL medium lacking Tryptophan (26.7 g/L minimal SD base, -Trp dropout supplement, 50 µg/mL kanamycin, pH 5.8) until they reached $OD_{600}$ equal to 0.4. Cells were then combined with the Y187 yeast strain transformed with the cDNA library and they were incubated at 30°C in 50 mL 2x YPDA liquid medium (20 g/L bacto yeast extract, 40 g/L bacto peptone, 40 g/L Glucose monohydrate, 80 mg/L Adenine hemi-sulphate, 50 µg/mL kanamycin, pH 6.5) in a 2 L flask at 25 rpm. 24 hours later 10 µL were removed to check for diploids (number of 8-like shaped cells) using a phase contrast microscope (40x objective). The culture was then centrifuged at 1000g for 10 mins and the pellet was washed with 0.5x YPDA liquid medium (5 g/L bacto yeast extract, 10 g/L bacto peptone, 10 g/L Glucose monohydrate, 20 mg/L Adenine hemi-sulfate, 50 µg/mL kanamycin, pH 6.5) and then resuspended in 10mL of 0.5x YPDA liquid medium. Finally, the whole culture was plated on 51 petri dishes of 150mm diameter (26.7 g/L minimal SD base, dropout supplement, 20 g/L agar, 50 µg/mL kanamycin, pH 5.8) lacking Adenine and Histidine and supplemented with 10 mM 3-AT (a HIS3 promoter inhibitor).

### 2.7.4 RNA isolation and cDNA production from P24 mouse brain

RNAs were isolated from P24 mouse brain tissue using Trizol (Invitrogen) according to the manufacture's protocol. All steps were carried on ice using ice-cold RNase-free 1.5 mL tubes and tips. All equipment and bench area used were

sprayed with RNaseZAP (Sigma). Briefly, brain tissue was homogenised using 10 volumes of Trizol reagent and an oven-baked glass-Teflon homogeniser. Homogenate was centrifuged at 12,000g for 10 mins at 4°C and the pellet was discarded. Subsequently 200mL of chloroform per 1 mL of supernatant was added and the samples were mixed thoroughly by inverting the tubes several times. The samples were then centrifuged at 12,000 g for 15 mins at 4°C and the aqueous phase was transferred in a clean tube. RNAs were then precipitated using equal volume of ice-cold isopropanol and 250 µg/mL of glycogen. The samples were again centrifuged at 12,000 g for 10 mins at 4°C. The pellet was washed with 75% ethanol – prepared with DEPC-treated water – and the RNA was finally resuspended in RNase-free water. After the RNA extraction, RNAs were treated with DNase according to the "RNase easy protocol" from Qiagen. The A260/280 ratio – that determines RNA quality – was measured using a NanoDrop 2000 UV-Vis Spectrophotometer (Thermo Scientific).

### 2.7.5 Cloning of Eps8 deletion constructs

Eps8-myc cloned onto the PCS2+ was a kind gift from Dr Miller JR (University of Minnesota, Minneapolis). To create the Eps8Δ180-584 the Eps8-myc was digested with EcoRV and the backbone was re-ligated, which resulted to the release of the 180-584 aa. To create the Eps8Δ1-585 the Eps8-myc was digested with EcoRV and ClaI, the 700bp fragment was ligated to pBluescript, which was then digested with EcoRI and XhoI and cloned back to the PSC2+ vector. The Eps8Δ1-366 construct was generated by a BamHI and BglIII digest of the Eps8-myc. The backbone was ligated, resulting in the deletion of the 1-366 aa. To create the Eps8Δ550-693 the Eps8-myc was digested with SacI. The two large DNA fragments were then ligated. To create the Eps8Δ533-821 the Eps8-myc was used as a template for a PCR reaction, Fwd: 5' – AAGTACATCGATATGAATGGTC ATATGTCTAACCGC – 3' and Rvs: 5' – AAGTACCTCGAGTCATAGGTCTTCGGAGATT AGCTTTTGCTCG – 3'. The PCR product was inserted onto PCS2+ using the ClaI and XhoI sites. A mutant Eps8 carrying three point mutations (V729A, T731A and W732A) called Eps8TM was created using the QuickChange Lightning Site-Directed Mutagenesis kit (Stratagene) following the manufacture's guidelines. Primers used were: Fwd: 5' – TCCTCACCGGAAGAAGCAAAGGCTGCGCTGCAGTC

AAAGGGA –3' and Rvs: 5'- TCCCTTTGACTGCAGCGCAGCCTTTGCTTCTTCCGGTG AGGA – 3'.

### 2.7.6 Small hairpin RNAs (shRNAs) against Eps8

shRNAs specific to both rat and mouse Eps8 were cloned into the pSuper vector (OligoEngine). Synthetic complementary DNA oligos were annealed and then cloned onto the BglII and HindIII sites of the pSuper vector. Each oligo contains the BglII site, a 21nt target sequence, a 9nt loop sequence (GTCAAGAGC), the 21nt target sequence in reverse compliment followed by a TTTTTTT termination sequence and the HindIII site. Target sequences were found using the "siRNA Target finder" from Ambion. shRNA#1: 5' – AGGCCCTTTATGAACAAAGTT – 3', shRNA#2: 5' – ACATGGATTCAACCTTCTGTT – 3' and shRNA#3: 5' – GAAATACGC CAAATCCAAGTT – 3'. Insertion of these oligos into pSuper vector destroys the BglII site thus making easier the identification of the correct clone. Clones were then sequenced to the insertion of the shRNA. A combination of the three shRNAs was used to achieve a significant level of knockdown, as assessed by the total levels of endogenous Eps8 in somas normalised to soma volume and the number of endogenous Eps8 puncta per dendritic length in hippocampal neurons.

## 2.8 Statistical analysis

Values given are mean ± error. Data presented is the pool from at least three independent experiments unless stated otherwise. For datasets with normal distribution, ANOVA test was used. For datasets with non-normal distribution, the Kruskal-Wallis test was used.

# Chapter 3:

# Eps8 is a direct interactor of Dvl1 in the brain

# 3.1 INTRODUCTION

The development of neuronal networks requires a combination of signalling pathways that induce changes in the structure and dynamics of the actin cytoskeleton. Wnt signalling factors regulate various aspects of neuronal development, from axonal and dendritic outgrowth to synapse formation and maintenance (Budnik & Salinas , Ciani & Salinas 2005, Rosso & Inestrosa 2013, Salinas , Salinas & Zou 2008). Previous studies have demonstrated that Wnt factors promote the remodelling of axonal growth cones by inducing actin dynamics (Hoyos-Flight, PhD thesis 2005) and the formation of dendritic spines (Ciani et al 2011, Farias et al 2009, Hiester et al 2013, Sharma et al 2013, Varela-Nallar et al 2010), actin-rich dendritic protrusions that receive excitatory inputs. However, the molecular mechanisms that are activated by Wnts to regulate the actin cytoskeleton during these processes remain elusive.

All Wnt signalling pathways described so far (Chapter 1.4) result in activation of the scaffold protein Dishevelled (Dvl), containing three conserved domains: an the DIX (Dishevelled, Axin) domain located in the N-terminus, a central PDZ (PSD95, Discs Large, Zonula occludens-1) domain and a C-terminal DEP (Dvl, Egl-10, Pleckstrin) domain (Gao & Chen 2010). Accumulating evidence show that Dvl associates and regulates the actin cytoskeleton in several ways. The first indication that Dvl regulates the actin cytoskeleton became evident from the first moment of its discovery (Fahmy & Fahmy 1959). Dvl was initially identified in Drosophila and took its name, because fly mutants for Dsh (the Drosophila Dvl homolog) had “dishevelled” wing hair (Fahmy & Fahmy 1959). The hairs in the fly wings are growing from actin-rich “pre-hair” structures after continuous actin polymerization at their distal ends. However, in Dsh mutant flies these “pre-hair” structures are mis-localized and their polarity is uneven resulting in a “dishevelled” appearance (Millar et al 1999). Later studies demonstrated that Dvl indeed can regulate the actin cytoskeleton through the activation of Rac and Rho small GTPases (Habas et al 2001, Rosso et al 2005). In addition, Dvl binds to actin stress fibers (Torres & Nelson 2000) and is localised on neuronal filopodia (Rosso et al 2005). Importantly, Dvl can directly bind to the actin cytoskeleton through its DIX domain (Capelluto et al 2002). These findings suggest that Dvl can regulate the actin cytoskeleton through direct and indirect pathways.

Previous studies have demonstrated that Dvl1 is required for Wnt-induced changes in the actin cytoskeleton during the formation of neuronal circuits. In axons, Wnt3a induces the terminal remodelling of NT3-rensponsive DRG neurons, a process manifested by growth cone enlargement and lamellar protrusion, through Dvl1 (Purro et al 2008). In addition, Dvl1 is required for Wnt7a signalling postsynaptically, as gain of function of Dvl1 induces spine growth through a pathway that involves local CaMKII activation, whereas neurons lacking Dvl1 do not respond to Wnt7a (Ciani et al 2011). However, the mechanism(s) by which Dvl1 affects actin cytoskeleton to promote growth cone remodelling and spine growth are not well understood.

Actin-binding proteins (ABPs) play key roles in actin organisation and dynamics by controlling filament assembly and disassembly, as well as organisation into actin superstructures (Chapter 1.5). Eps8 (epidermal growth factor receptor pathway substrate 8) is a multi-functional actin-binding protein that directly interacts with Dvl1 (Inobe et al 1999). Eps8 posses an intrinsic capping and bundling activity and in addition participates in tyrosine receptor-mediated Rac1 activation (Disanza et al 2004, Disanza et al 2006, Hertzog et al 2010, Innocenti et al 2002, Offenhauser et al 2004, Offenhauser et al 2006, Scita et al 1999). Therefore, Eps8 modulates both actin dynamics and organisation through direct and indirect mechanisms. In neurons, Eps8 is prominently enriched in axonal growth cones, where it regulates filopodium formation through its capping activity (Menna et al 2009). However, its role on axonal terminal remodelling and spine formation remains elusive.

The aim of this chapter was to identify – through an unbiased screen – molecules that interact directly with Dvl1 and can act as downstream effectors in Wnt-mediated actin rearrangements during axonal remodelling and spine morphogenesis. We demonstrate that Dvl1 directly interacts with the actin-binding protein Eps8 in the adult mouse brain through its PDZ domain. We have identified the domain in Eps8, which is important for this interaction, and found that it lies between amino acids 181-366. Our findings suggest that Eps8 could be a Dvl1 effector during Wnt-mediated axonal remodelling and spinogenesis.

# 3.2 RESULTS

## 3.2.1 Yeast-two hybrid screen.

Given that Dvl mediates the effect of Wnts on axon remodelling and spine morphogenesis, we aimed to identify proteins that interact with Dvl that could mediate these process. With this in mind, we performed a yeast two-hybrid screen (Y2H) using as bait the full-length sequence of Dvl1. Yeast two-hybrid screen is a powerful technique, which is based in the intrinsic properties of the transcription factor GAL4 (Fields & Sternglanz 1994, Young 1998). GAL4 contains two separate domains important for its DNA binding (BD) on upstream activating sequences (UAS) and its ability to activate transcription (AD) (Figure 3.1A). This system provides a sensitive method to examine direct interaction between two proteins by fusing each protein to the BD and AD domains of GAL4, respectively (Figure 3.1B). To discover novel direct interactors that could modulate Dvl1 function during the formation and maturation of neuronal connections, we used a commercially available adult mouse brain cDNA library (Clontech). Dvl1 was cloned into the pGBDT7 vector that contains the BD domain of GAL4, whereas the clones present in the cDNA library were already inserted to the pGADT7 vector that contains the AD domain of GAL4 (Figure 3.1C).

Yeast two hybrid screens are simple, rapid and inexpensive, with the disadvantage of producing a significant number of false-positive interactions. To overcome this limitation, we performed a high-stringency nutrition selection by using the Y187 yeast strain which contains 3 different reporter genes: HIS3, ADE2 and MEL1 (Figure 3.1C). These genes encode for histidine and adenine (two essential amino acids) and α-galactosidase, a secreted enzyme that can be assayed directly using X-α-Gal. Selecting for all three reporter genes will substantially minimize the identification of false-positive candidates and select molecules that have strong interaction with the target molecule in this case Dvl1.

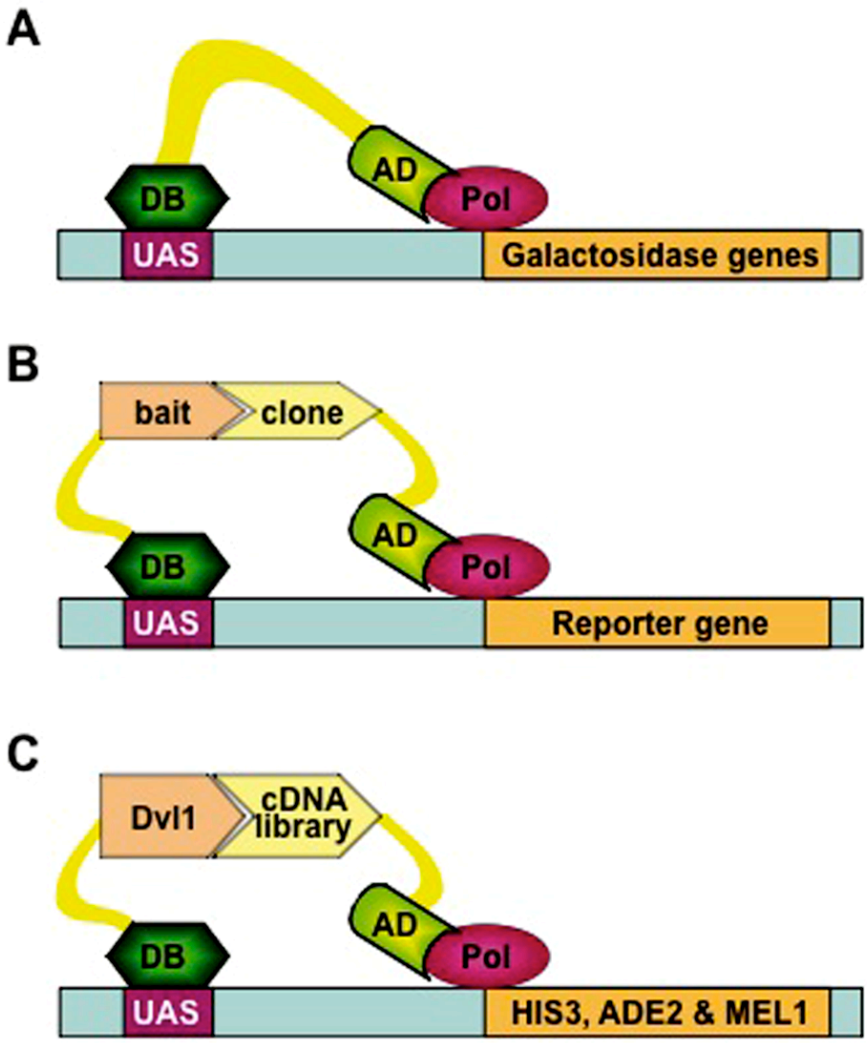
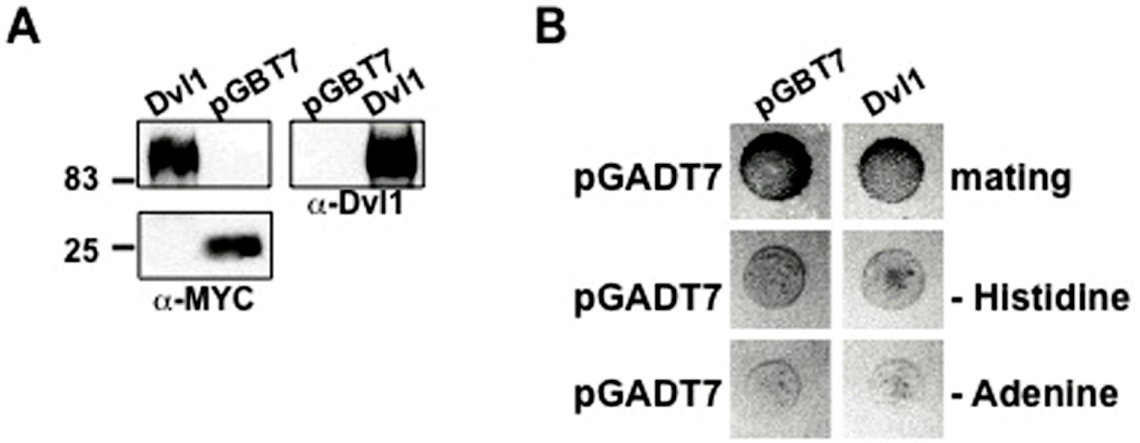



**Figure 3.1: Illustration of the Yeast two-hybrid technique.** The transcription factor GAL4 possesses a DNA-binding domain – which binds to UAS – and an Activator domain – which binds to the RNA polymerase and activates transcription of galactosidase genes (A). These domains are used in the yeast-two hybrid system to detect direct interactions between two proteins (B). To discover novel direct interactors of Dvl1 we fused its full-length sequence to the DNA-binding domain and we used it as a bait in a screen using a commercial adult mouse brain cDNA library (C).

### 3.2.2 Dvl1 does not posses an intrinsic transcriptional activity.

Before performing the screen, we first verified that our bait (full length Dvl1) is stably expressed in yeast by isolating protein extracts from yeast transformed with Dvl1 (Figure 3.2A). In addition, we tested whether Dvl1 has any intrinsic ability to activate transcription of the genes that will be used for selection (HIS3 and ADE2). We found that yeast cells transformed with Dvl1 do not grow in medium lacking histidine or adenine, thus Dvl1 does not activate the

transcription of selection genes (Figure 3.2B). Finally, we examined whether our bait induces cell toxicity in yeast by testing the cell growth of yeast transformed with Dvl1 after an overnight culture, which was then diluted to reach $OD_{600}$ equal to 0.2 and retested after two hours (Figure 3.2C). Together these results demonstrate that full length Dvl1 is a suitable molecule to be used as bait in our yeast two-hybrid screen.




C

| vector | $OD_{600}$ after 18hrs | $OD_{600}$ after 2hrs |
|---|---|---|
| - | 3.5 | 0.68 |
| pGBDT7 | 1.5 | 0.42 |
| pGBDT7-Dvl1 | 2.2 | 0.32 |

**Figure 3.2: Dvl1 is a suitable bait for a yeast-two hybrid screen.** Dvl1 is properly expressed in yeast cells and it is not targeted for degradation (A). Dvl1 does not have any intrinsic transcriptional activity (B). Dvl1 neither inhibits cell growth nor induces cell toxicity in yeast cells (C).

### 3.2.3 Screening for Dvl1 direct interactors

To introduce the pGBT7-Dvl1 vector and the cDNA library clones we performed yeast mating. The cDNA library was already transformed into yeast strain Y187 when purchased, thus we transformed the pGBT7-Dvl1 vector onto the AH109 strain, which is the mating partner of the Y187 strain. We next performed mating between these two haploid strains. Our mating efficiency was 5.1% after 24 hrs, which was sufficient for downstream applications. Hence, the mating was stopped and the whole culture was plated on high-stringency nutrition selection plates, lacking adenine and histidine. In total, we obtained 316 colonies, which were then transferred onto plates containing X-α-Gal, lacking adenine and

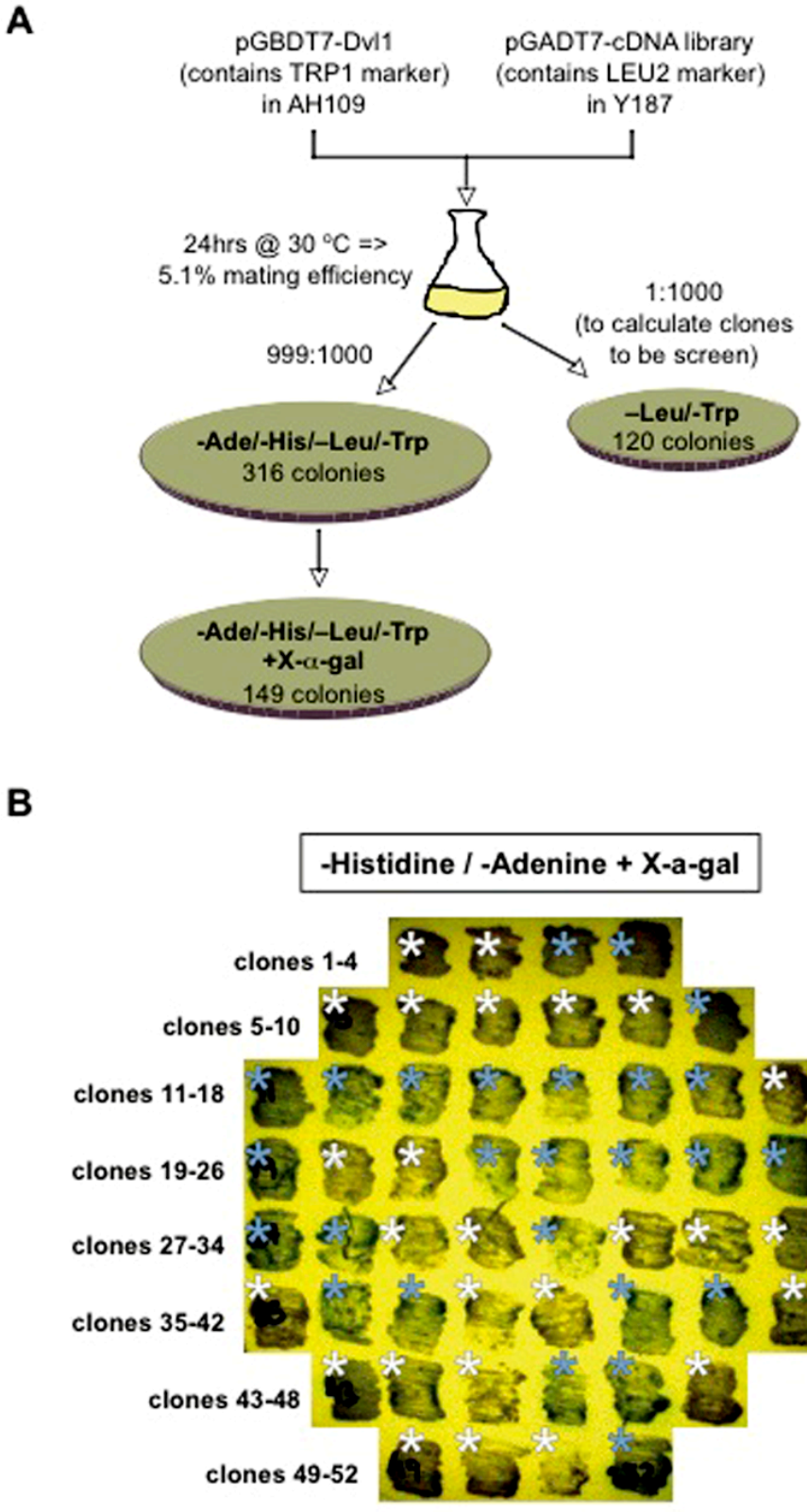



**Figure 3.3: Yeast-two-hybrid screen overview.** Yeast cells (AH109) expressing Dvl1 were mated with cells (Y187) expressing the cDNA library. The culture was then plated on high-stringency nutrition selection plates, lacking both adenine and histidine. In total 149 clones, which were positive for all reporter genes, were selected for further screening (A). Example of a high-stringency nutrition plate containing X-α-gal (B). Asterisks represent our classification to blue and white colonies.

histidine (Figure 3.3A). The majority of the colonies grew again in fresh plates and 149 of them became blue in the presence of X-α-Gal (Figure 3.3B). We therefore focused on the blue colonies, as these colonies passed the triple selection process and represent high confidence interactions.

During library construction and mating procedures some of the clones may be over-represented in our sample. To avoid duplicates and speed up the process of validation, we isolated plasmid DNA from all candidate clones and PCR-amplified the cDNA clones (Figure 3.4A), using the Matchmaker 5' and 3' screening set of primers that flank the cDNA insert site in the pGADT7 vector. These PCR products were digested with AluI to examine the DNA profile of the clones (Figure 3.4B). Based on the insert size and the AluI digest profile, we identified several identical clones. For verification purposes, we set up another digest using HaeIII for selected clones (Figure 3.4C). Using this approach, we identified 37 repeated candidates that were carrying identical clones, resulting in 112 potential unique clones.

Before DNA sequencing, we first tested whether the clones we retrieved bear any intrinsic transcriptional activity and whether their interaction with Dvl1 can be reproduced. Since the plasmid DNA isolated from yeast is a mixture of pGBDT7 vector (containing Dvl1) and pGADT7 vector (containing a library clone), we transformed *E. coli* and use ampicillin selection to select for the pGADT7 vector, which carries an ampicillin-resistance gene. Each clone was subsequently transformed back to the Y187 yeast strain and mated with empty pGBDT7 vector or pGBDT7-Dvl1 (Figure 3.5A). Using a high-stringency selection, we identified 19 clones that have a false-positive or negative interaction with our bait (Figure 3.5B), finally we continued the screening with 93 clones.

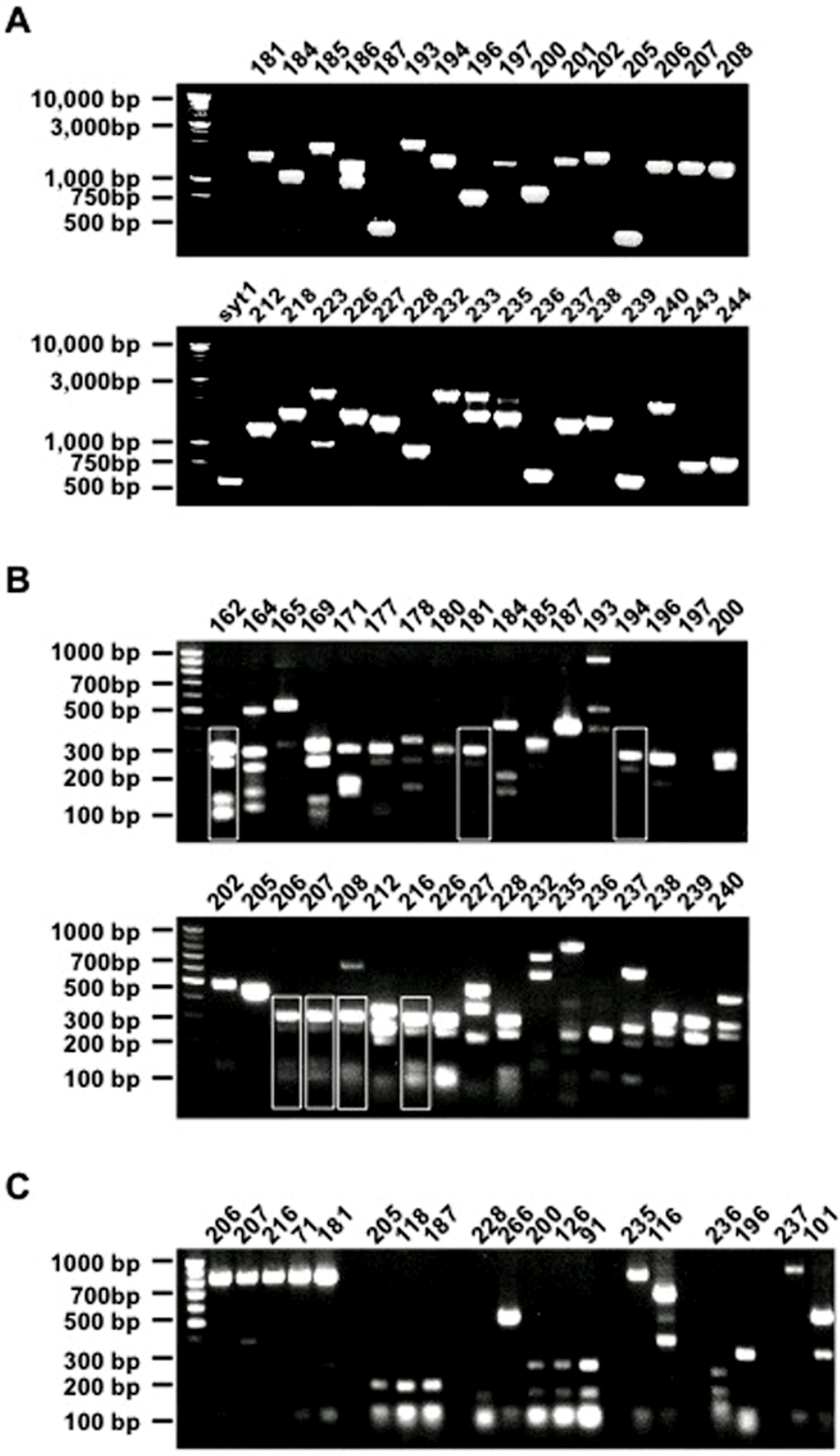



**Figure 3.4: Screening for duplicated clones according to their size and restriction profile.** Plasmid DNA from all blue colonies was isolated and used in a PCR reaction to amplify the cDNA insert (A). The PCR products were then cut using the frequent-cutter restriction enzymes AluI (B) and HaeIII (C). According to the size and the digest profile, we identified several identical clones. White boxes represent a clone which was present in several clones.

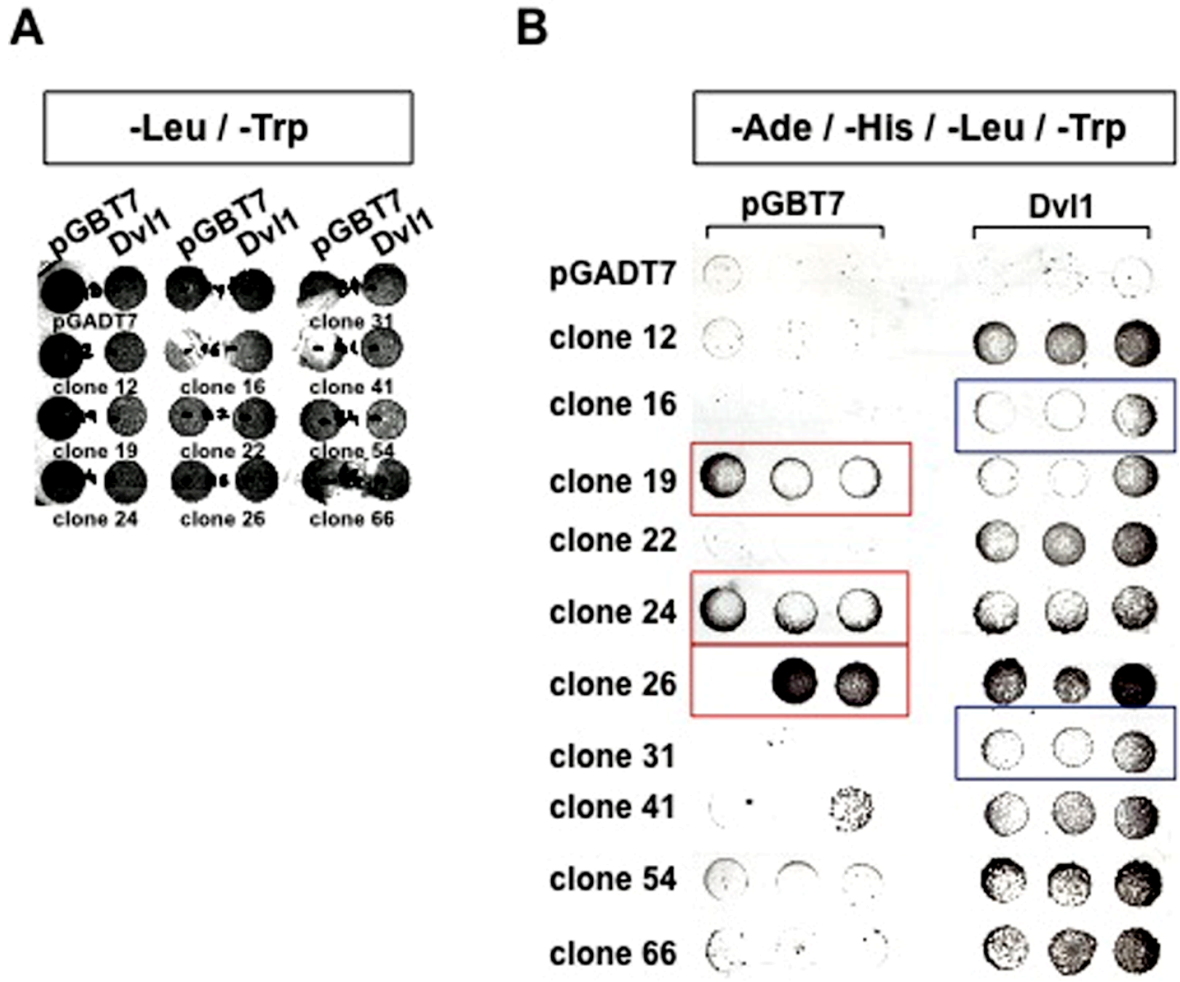



**Figure 3.5: Confirmation of the interaction and test for transcription auto-activation of the clones through yeast-two hybrid assays.** Plasmid DNA containing the cDNA from the library was isolates from all clones and was introduced again in yeast to test for a positive interaction with Dvl1. The yeast mating was successful since all clones grew in –Leu/-Trp selection (A). Using a high-stringency selection (B), we identified clones that either activate the reporter genes in the absence of Dvl1 (red boxes) or the interaction was not confirmed (blue boxes).

### 3.2.4 Identification of Dvl1 partners

To identify the genes representing each clone DNA sequencing was performed and the results were aligned to the mouse genome using the BLAST program (NCBI). We identified a number of published Dvl partners, serving as controls for this screen (Inobe et al 1999, Kishida et al 2007, Zhang et al 2006) and providing confidence that the screen was successful (Table 3.1). In addition to the already known interactions, we identified several novel potential interactors (Table 3.1). The remaining clones (27) were identified as genomic or 3' untranslated regions. Our attention was focused on Eps8L3, which is a member of the Eps8 family known for their multiple roles in actin cytoskeleton dynamics.

To verify the interaction of Eps8L3 with Dvl1, we cloned its full-length cDNA, which was PCR amplified of cDNA isolated from P24 mouse brain, onto the pGADT7 vector. We then performed a yeast two-hybrid assay using full-length Dvl1 or the PDZ domain of Dvl1 cloned onto the pGBDT7 vector (Figure 3.6A). We found that Eps8L3 interacts with Dvl1 through its PDZ domain, as it was previously reported for the main member of the Eps8 family (Inobe et al 1999). Since there were no commercial anti-bodies against Eps8L3, we continued our studies focusing on Eps8, which is the most highly expressed family member in the brain (Offenhauser et al 2006).

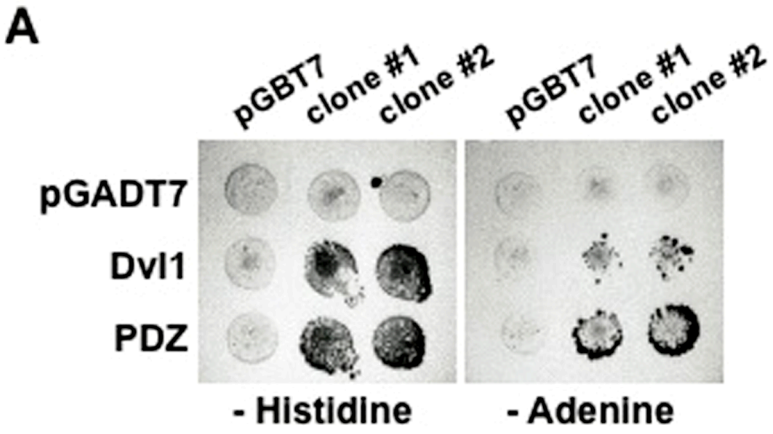



**Figure 3.6: Eps8L3 interacts with the PDZ domain of Dvl1.** Eps8L3 (two individual colonies #1 and #2) illustrates a strong interaction with full length Dvl1 or the PDZ domain of Dvl1, but not with the control vector (pGADT7). The interaction with PDZ is stronger, as it is represented by the -Adenine selection.

| Interactor | Times found | Novelty |
|---|---|---|
| stomatin like protein 2 | 1 | Yes |
| G gamma 3 | 5 | Yes |
| eps8l3 | 1 | (Inobe et al., 1999) |
| dapper homolog 3 | 3 | (Zhang et al., 2006) |
| microspherule protein 1 | 1 | Yes |
| psmd8 | 19 | Yes |
| sialyltranferase | 1 | Yes |
| synaptotagmin IV | 1 | Yes |
| synaptotagmin XI | 3 | (Kishida et al., 2007) |
| neuraminidase I | 21 | Yes |
| hypothetical protein LOC58520 | 2 | Yes |
| similar to OTTHUMP-0000002869 | 1 | Yes |
| zinc finger, SWIM domain containing 3 | 1 | Yes |
| PHD finger protein 20-like 1 | 1 | Yes |
| hypothetical protein LOC225929 | 1 | Yes |

**Table 3.1: Interactors of Dvl1 found in the yeast two hybrid screen.** Only the strongest interactors according to high stringency of selection conditions were chosen. Colonies: Number of individual colonies found for each interactor. Novelty: Refers to already published interactions.

### 3.2.5 Eps8 interacts directly with Dvl1 *in vivo* through 181-366aa

To examine whether Eps8 and Dvl1 interact in neuronal cells, we co-expressed Eps8 and Dvl1 in differentiated Neuroblastoma 2a (NB2a) cells that have neuronal characteristics. When Eps8 is expressed alone, it has a membrane-like localisation. However, in the presence of Dvl1, Eps8 adopts a punctuated distribution and co-localizes with Dvl1 puncta (Figure 3.7A). We then examined whether Eps8 interacts with Dvl1 by performing immunoprecipitation assays *in vitro* using in NB2a cell lysates (Figure 3.7B) and *in vivo* using total homogenate

from P24 mouse brain (Figure 3.7C). Collectively, these results demonstrate that Eps8 interacts with Dvl1 *in vivo*.

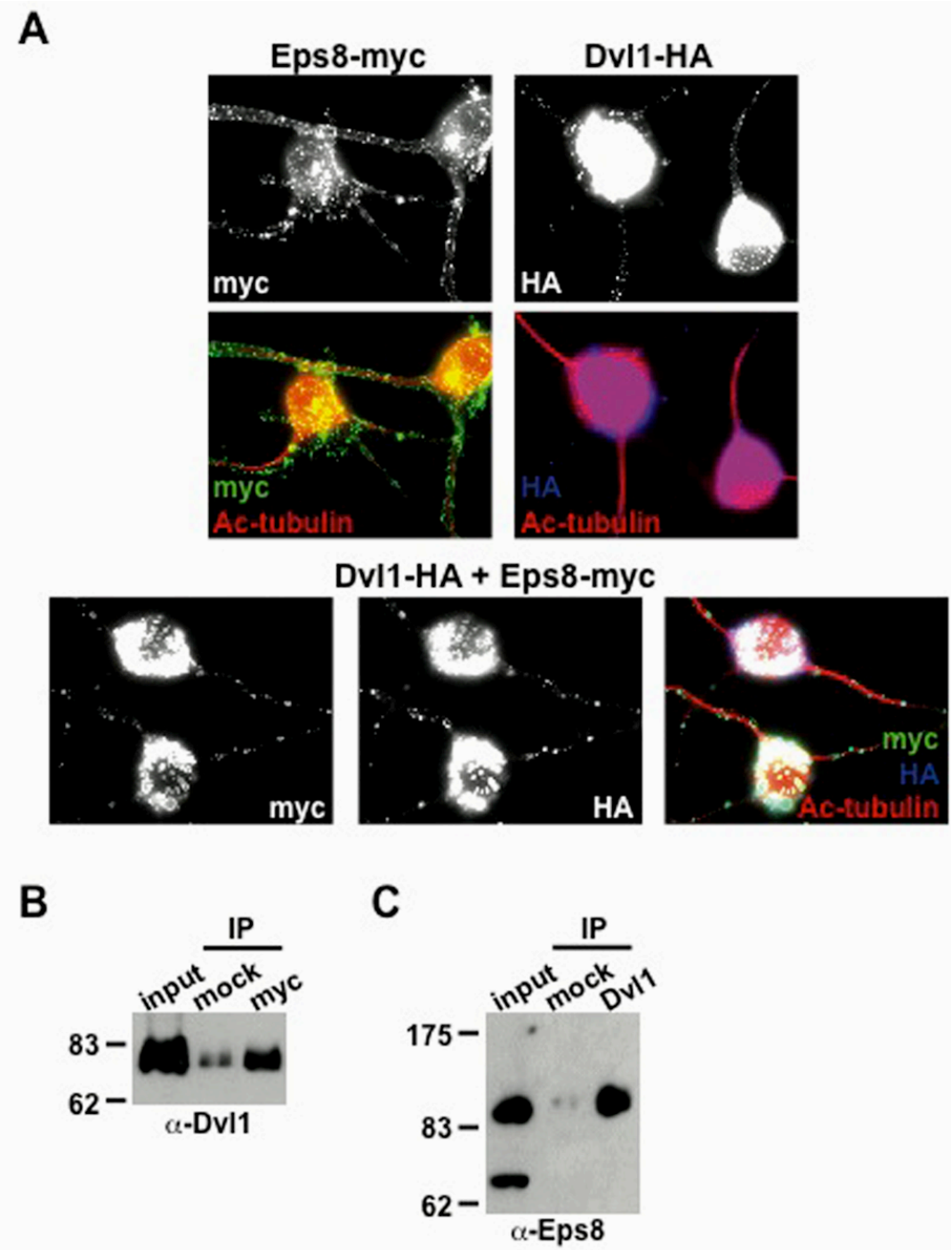



**Figure 3.7: Eps8 interacts with Dvl1 *in vivo*.** Expression of Dvl1-HA changes the cellular distribution of Eps8-myc into Dvl-like puncta in NB2a cells (A). Scale bar: 10μm. (B) Co-immunoprecipitation of Dvl1 by Eps8 (anti-myc) in NB2a cells that express Eps8-myc and Dvl1-HA. (C) Interaction of Eps8 with Dvl1 exists also in vivo, since Eps8 is co-immunopercipitated with Dvl1.

To identify which domain of Eps8 is required for the interaction with Dvl1, we created several truncated forms of Eps8 (Figure 3.8A). We expressed these

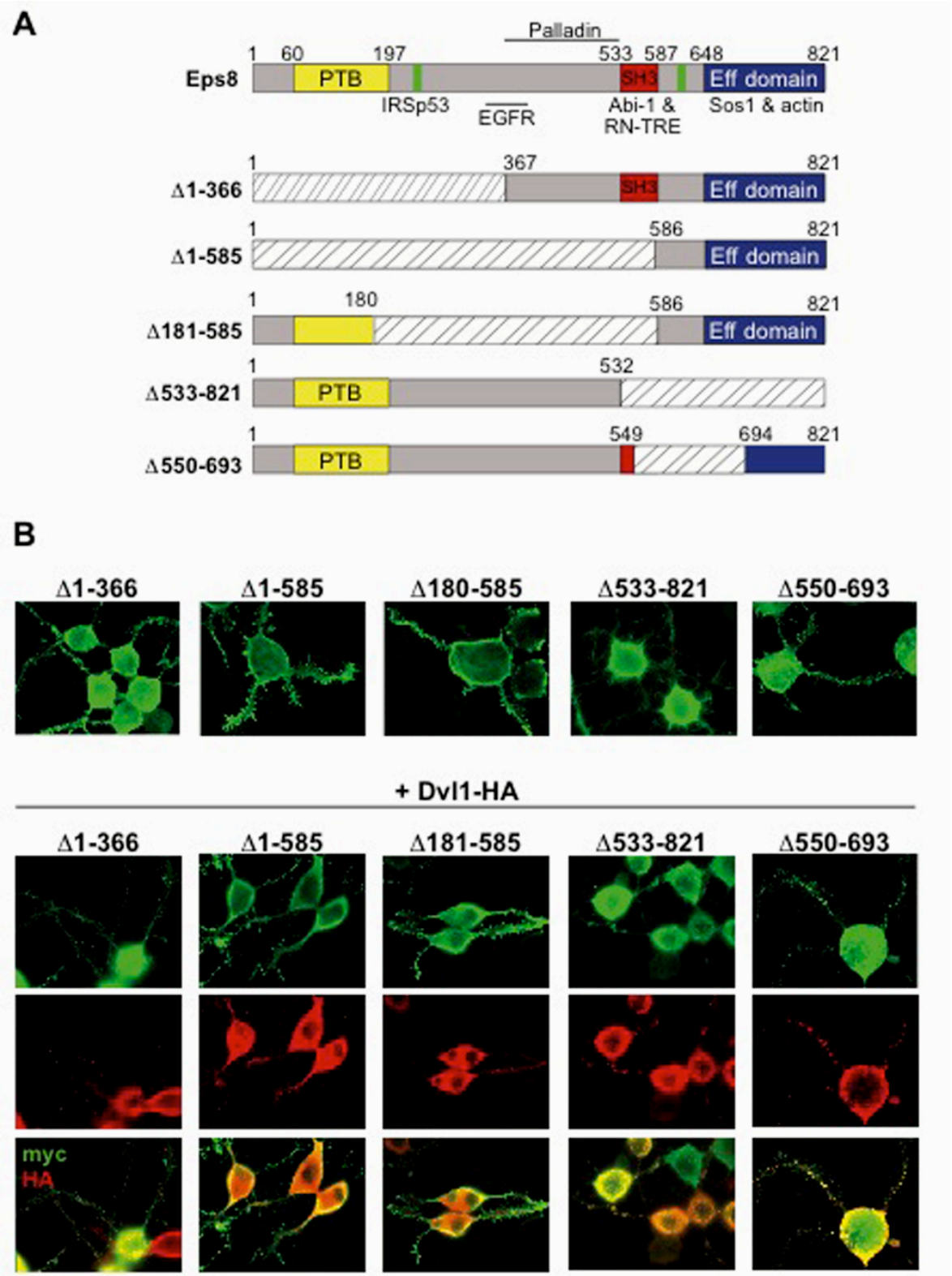



**Figure 3.8: Identification of the domain in Eps8 that interacts with Dvl1.** (A) Illustration of the Eps8 deletions designed and used to identify the domain that is required for the interaction with Dvl1. (B) Expression of Dvl1-HA with the deletions in (A) in NB2a cells shows that only Δ533-821 and Δ550-693 are still showing an interaction. Scale bar: 10μm.

truncated forms in NB2a cells together with full-length Dvl1 (Figure 3.8B) and we took advantage of the ability of Dvl1 to change the distribution of Eps8 (Figure 3.5A). From all 5 constructs tested, only two show a change in their localisation in presence of Dvl1 (Δ533-821 and Δ550-693). The remaining three: Δ1-366, Δ180-584 and Δ1-585 have a different pattern of localisation from Dvl1, which shows that the interaction with Dvl1 has been abolished (n=2, 10-20 cells per condition). We thus conclude that the domain of Eps8 that is required for the interaction with Dvl1 lies between amino acids 181-366 (Figure 3.9).

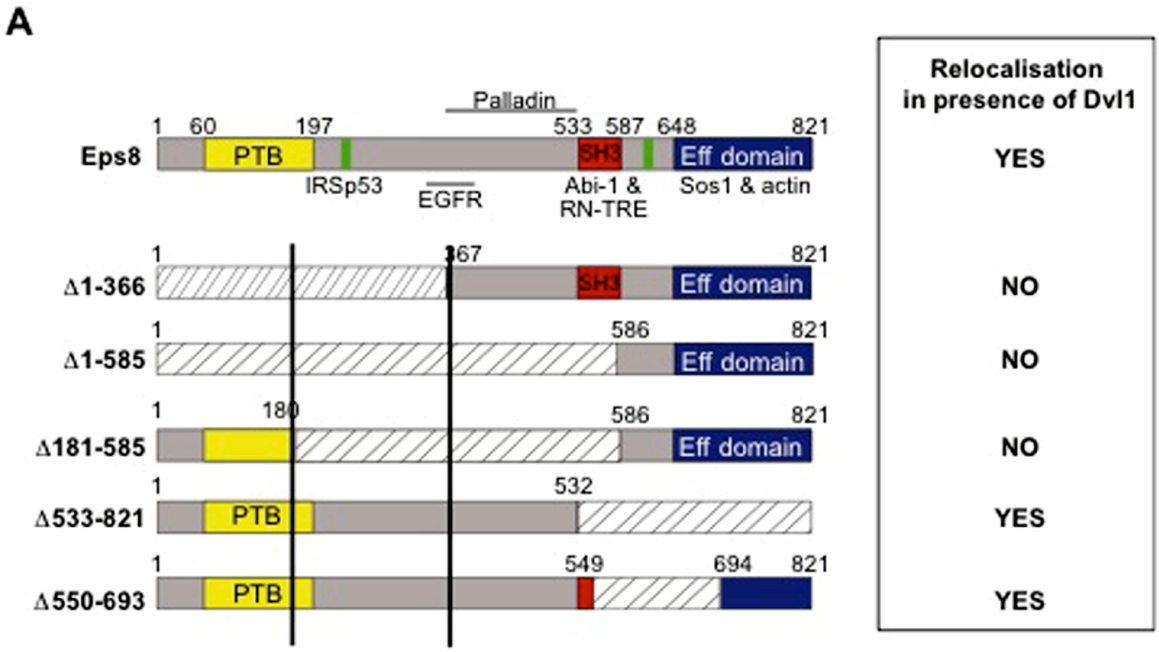



**Figure 3.9: Eps8 interacts with Dvl1 through amino acids 181-366.** (A) Based on the data provided in Figure 3.8, the domain in Eps8 that is required for its interaction with Dvl1 contains a Proline rich motif (green box), which is important for Eps8 interaction with IRSp53. In addition in this region there is the binding site for the EGF receptor.

## 3.3 DISCUSSION

Dvl is a scaffold protein involved in all Wnt signalling responses (Gao & Chen 2010, van Amerongen , Wharton 2003, Wynshaw-Boris). Although great process has been made in identifying the molecules that participate in the Wnt signalling pathways, there are a lot of missing links in our understanding of how Wnts modulate the actin cytoskeleton. Here we show that Eps8, an actin-binding protein, is a direct interactor of Dvl1 in the brain. We identified that amino acids 181-366 in Eps8 are important for its interaction with Dvl1. This region contains a Proline Rich (PR) motif, which is important for the interaction of Eps8

with the actin-bundling protein IRSp53 and part of the binding site for the EGF receptor (EGFR).

Dvl1 and Eps8 interaction was previously found through a yeast-two hybrid screen (Inobe et al 1999). In that study, authors showed that Dvl1 inhibits the EGFR-mediated tyrosine phosphorylation of Eps8 in heterologous cells (Inobe et al 1999). These results suggest that Dvl1 may regulate the binding of Eps8 interaction with the EGF receptor by delocalizing Eps8 from the cell membrane and sharing the same binding domain with EGF receptor. This could lead to attenuation of Rac-mediated responses, since Eps8 is a substrate of EGF signalling that activates Rac (Lanzetti et al 2000). Our results support this hypothesis, as we found that Dvl1 changes the localization of Eps8 in NB2a cells, which in the absence of Dvl1 has a membrane-like distribution.

In addition to Eps8, we also identified other novel interactors of Dvl1. These include Stomatin-like 2, Dact3 and MCSR1. StomL2 (Stomatin- like 2) is a member of the stomatin family (Owczarek et al 2001) been involved in touch sensation in mouse (Wetzel et al 2007) and C.elegans (Huang et al 1995). The mechanism that was described involved its interaction with the cytoskeleton and ion channels. Also, members from the same family have been found to regulate acid-sensing ion channel gating (Price et al 2004). Importantly, StomL2 is present in sensory neurons in dorsal root ganglia (Mannsfeldt et al 1999) and in the postsynaptic density fraction (PSD) of forebrain synaptosomal preparations (Satoh et al 2002) and interacts with voltage-gated calcium channels (Davies et al 2006). It would be interesting to test whether this protein is a modulator of Wnt signalling during spine development.

Dact3 (dapper, antagonist of ß-catenin, homolog 3) is an antagonist of Wnt signalling. Dapper can inhibit Wnt-mediated responses in different aspects. Initially, it was discovered as a molecule that promotes β-catenin degradation and inhibits JNK activation (Cheyette et al 2002). In particular, it was shown that Dapper is required for basal cell function, repression of β-catenin accumulation and JNK signalling (Cheyette et al 2002). Later, it was demonstrated that Dapper can also induce Dvl degradation (Zhang et al 2006). Although, the function of the dapper family in regulating Wnt signalling is well known, their role in neuronal connectivity had not been studied until recently.

Studies in hippocampal neurons demonstrated that Dact1 (dapper, antagonist of β-catenin, homolog 1) is required for dendritic arborization and spine formation *in vivo* through Rac activation (Okerlund et al 2010). It will be interesting to test whether Dact3, which is the most abundant family member in the adult mouse brain (Fisher et al 2006), has similar function in postsynaptic development and whether it participates in Wnt-mediated spinogenesis.

Microspherule protein 1 (MCRS1) is another molecule identified as a Dvl1 direct interactor from our screen. Interestingly, Dvl2 has been shown to bind with MCRS1 (Rual et al 2005). MCRS1 is a RNA-binding protein, which has been shown to interact with FMRP (fragile X mental retardation protein) in polyribosomes isolated from synaptosomal preparations (Davidovic et al 2006). Interenstingly, FMRP is an important regulator of local mRNA translation in dendrites (Bagni & Greenough 2005, Grossman et al 2006, Zalfa et al 2006). Mice and humans lacking FMRP possess a higher density of immature dendritic spines (Beckel-Mitchener & Greenough 2004, Irwin et al 2000, Portera-Cailliau 2012). The finding that Dvl1 directly interacts with MCRS1, a protein that itself is a direct interactor of FMRP, suggests that Dvl1 might associate with FMRP to regulate spine growth through changes in local translation. This interaction was verified by another PhD student in the lab and will not be discussed further in this thesis.

In summary, we identified several molecules that can be potential interactors of Dvl1 during the formation of neuronal circuits. We focused our attention to Eps8, a protein with multiple functions in actin dynamics. We found that the PDZ domain of Dvl1 and amino acids 181-366 of Eps8 are important for their interaction. We also show that Eps8 is a partner of Dvl1 *in vivo*. In the following two chapters we are going to examine whether there is any functional interaction between Eps8 and Dvl1 during axonal remodelling and spine growth.

# Chapter 4:

# Role of Eps8 in Wnt-mediated axonal remodelling

# 4.1 INTRODUCTION

The arrival of axons at their synaptic targets results in extensive remodelling of the growth cone and the terminal portions of the axon leading to the formation of terminal branches and presynaptic boutons. This extensive remodelling is crucial for the formation of functional neuronal circuits and requires coordinated changes in the organisation and dynamics of both the actin and microtubule cytoskeletons (Dent et al 2011, Geraldo & Gordon-Weeks 2009, Gomez & Letourneau 2014, Lowery & Van Vactor 2009, Vitriol & Zheng 2012). Target-derived cues promote terminal remodelling of axons, but little is known about how these extracellular signals influence the cytoskeleton.

Wnt secreted molecules play important roles in the formation of neuronal circuits by regulating axon pathfinding and remodelling, dendritic development and synapse assembly (Budnik & Salinas 2011, Ciani & Salinas 2005, Koles & Budnik 2012, Mulligan & Cheyette 2012, Park & Shen 2012, Salinas 2012). Wnts act as target-derived signalling molecules that promote axon terminal remodelling and the subsequent assembly of presynaptic boutons (Hall et al 2000, Krylova et al 2002, Purro et al 2008. In the cerebellum, Wnt7a released by granule cell neurons acts on incoming mossy fiber axons to induce growth cone enlargement and axonal spreading, processes that are accompanied by the recruitment of presynaptic components {Hall, 2000 #618). Importantly, Wnt7a deficient mice exhibit defects in axonal terminal remodelling and the accumulation of synaptic proteins at mossy fibre axons (Ahmad-Annuar et al 2006, Hall et al 2000). In the fly neuromuscular junction (NMJ), Wg, another member of the Wnt family, is required for the proper formation of synaptic boutons (Packard et al 2002). In the spinal cord, motorneuron-derived Wnt3 promotes the axonal terminal remodelling of NT-3 responsive dorsal root ganglia (DRG) neurons (Krylova et al 2002). Therefore, Wnt proteins are target-derived signals that induce extensive structural remodelling of presynaptic axonal terminals.

During axon remodelling, Wnts induce profound changes in the organisation of microtubules (MTs). In the presence of Wnts, MTs extend towards the leading edge of the growth cone, but their direction is severely affected resulting in the formation of looped MTs (Hall et al 2000, Purro et al 2008). Wnt3a acts through a

divergent canonical ß-catenin pathway that is independent of transcription, but requires Dishevelled-1 (Dvl1) and Glycogen synthase kinase 3β (Gsk3ß) inhibition to regulate MT looping (Purro et al 2008). This pathway directly signals to the cytoskeleton by inducing loss of APC from the MT plus-ends, resulting in defects in the directionality of MT growth (Purro et al 2008). Importantly, studies at the Drosophila NMJ revealed that the divergent canonical Wnt pathway through Shaggy/Gsk3 promotes axonal remodelling manifested by the formation of satellite boutons and the presence of looped microtubules (Franco et al 2004, Miech et al 2008). Consistently, wg mutants have defects in synaptic bouton formation and morphology (Miech et al 2008, Packard et al 2002). Together, these studies demonstrate that Wnt signalling factors target the microtubule cytoskeleton to drive axons to their synaptic targets. However, the effects of Wnts on axonal morphology, such as axonal spreading and growth cone enlargement, suggest that Wnts also modulate the actin cytoskeleton.

Actin dynamics is important for proper axonal extension and axon guidance responses (Gomez & Letourneau 2014, Hall & Lalli 2010, Kalil & Dent 2005, Lykissas et al 2007), however during axonal remodelling the changes in the actin cytoskeleton are not well understood. Detailed examination of the actin cytoskeleton during Wnt3a-mediated axonal remodelling revealed an accumulation of F-actin in growth cones within 15 mins after Wnt3a application (Hoyos-Flight, PhD thesis 2005). F-actin recovery experiments after Cytochalsin D (CytoD) treatment, a pharmacological inhibitor of actin assembly, showed that Wnt3a induces actin polymerisation (Figure 4.1A; Hoyos-Flight, PhD thesis 2005). Intriguingly, CytoD application in Wnt3a-treated cones induced a significant decrease in F-actin accumulation within the first 2 mins of application, indicating that Wnt3a promotes the assembly of highly dynamic actin filaments that are quickly dissembled in the presence of CytoD (Figure 4.1B; Hoyos-Flight, PhD thesis 2005). Moreover, time-lapse recordings of DRG neurons expressing GFP-actin showed that Wnt3a promotes lamellar protrusion and enhances filopodia movement speed in growth cones (Hoyos-Flight, PhD thesis 2005), processes that are both mediated by increased actin dynamics. Altogether these results indicate that Wnt3a induces the formation of highly dynamic actin filaments.

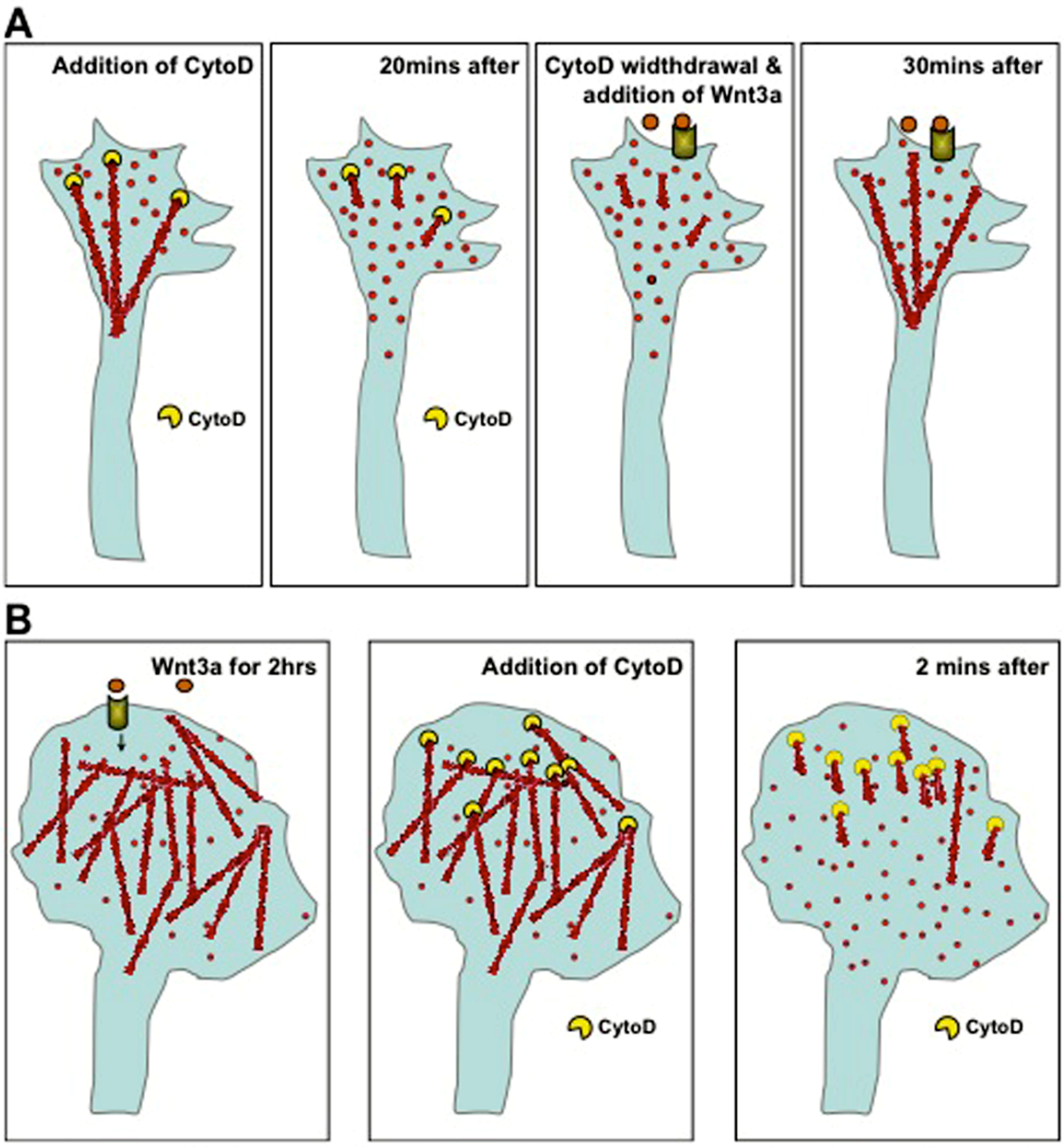



**Figure 4.1: Wnt3a promotes actin dynamics.** (A) After CytoD treatment, the level of F-actin in axonal growth cones is recovered faster in Wnt3a-treated growth cones in comparison to control-treated cells, indicating that Wnt3a promotes actin polymerization. (B) CytoD causes a greater decrease in F-actin area in growth cones of Wnt3a-treated neurons than controls, demonstrating that Wnt3a induces actin depolymerization.

The actin cytoskeleton is regulated by a number of actin-binding proteins (ABPs) that control nucleation, severing, cross-linking, and capping of actin filaments, as well as monomer sequestering. Although a large number of ABPs are present at growth cones, only few have been examined in axon guidance and target recognition (Dent et al 2011, Gomez & Letourneau 2014, Ishikawa & Kohama 2007, Pak et al 2008). Eps8 (epidermal growth factor receptor pathway substrate 8) is a multi-functional actin-binding protein that regulates the actin cytoskeleton through diverse mechanisms (Disanza et al 2004, Disanza et al 2006, Offenhauser et al 2006, Scita et al 1999). Eps8 binds to filamentous actin

and directly modulates actin dynamics through its barbed-end capping and bundling activities (Disanza et al 2006, Hertzog et al 2010). Eps8 can also regulate the actin cytoskeleton indirectly via tyrosine receptor-mediated Rac1 activation (Innocenti et al 2002, Offenhauser et al 2004, Scita et al 1999). Therefore, Eps8 modulates both actin dynamics and organisation. In neurons, Eps8 is prominently enriched in axonal growth cones and dendritic spines where it regulates filopodium formation through its capping activity (Menna et al 2009). However, its role on axonal terminal remodelling remains elusive.

The aim of this chapter was to dissect the pathway(s) by which Wnts induce changes to the actin cytoskeletal to regulate axon remodelling. As revealed by our yeast-two hybrid screen (Chapter 3), the actin-binding protein Eps8 is a direct interactor of Dvl1, we thus decided to examine whether it is involved Wnt-mediated actin cytoskeletal changes. In summary, we show that expression of Dvl1 or Gsk3β inhibition mimic the effect of Wnt3a in F-actin accumulation. Importantly, we demonstrate that gain of function of Eps8 mimics Wnt3a-mediated growth cone remodelling, whereas loss of function of Eps8 impairs Wnt3a-induced axonal remodelling. Our studies identify Eps8 as a novel target for Wnt signalling in the regulation of the actin cytoskeleton during axonal remodelling.

# 4.2 RESULTS

## 4.2.1 Wnt3a modulates axonal actin through Dvl1 and Gsk3β

To determine how Wnt3a regulates actin dynamics, we first examined the role of Dvl1 and Gsk3β as these two Wnt signalling components contribute to Wnt-mediated axonal remodelling (Purro et al 2008). Through gain of function experiments in dorsal root ganglia (DRG) neurons, we found that Dvl1 significantly increases F-actin accumulation in growth cones (Figure 4.2A-C, cnt: 40; Dvl1: 46 growth cones). Next, we examined whether Gsk3β inhibition promotes accumulation of F-actin (Figure 4.2D). Using a cell-permeable peptide inhibitor that specifically blocks Gsk3β (Plotkin et al 2003), we found a significant increase in F-actin accumulation in DRG growth cones (Figure 4.2E and 4.2F, cnt: 125; Gk3β inh: 91 growth cones). Together, these results suggest that Wnt3a regulates actin dynamics through Dvl1 and inhibition of Gsk3β.

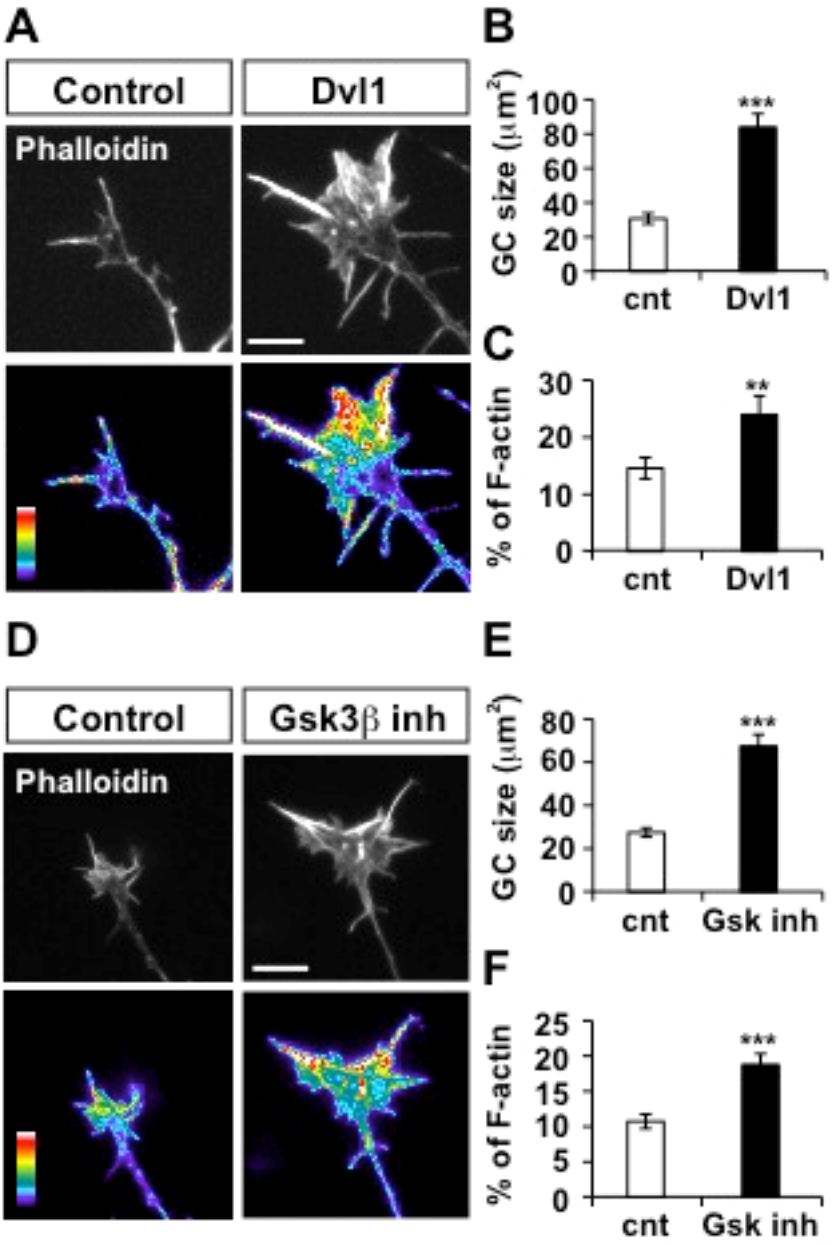



**Figure 4.2: Dvl1 and inhibition of Gsk3β mimic the effect of Wnt3a in F-actin accumulation.** Dvl1 induces F-actin accumulation in growth cones (A). Pseudocolor images of growth cones labelled with phalloidin highlight the differences in F-actin levels between Dvl1 and control GFP-expressing cells. Scale bar: 5μm. Dvl1 promotes growth cone enlargement (B), but also induces the percentage of growth cone area with bright F-actin fluorescence (C). A specific Gsk3β peptide inhibitor promotes accumulation of F-actin in growth cones (D). Scale bar: 5μm. Inhibition of Gsk3β induces axonal remodelling (E) and increases the percentage of growth cone area with bright F-actin fluorescence (F). **p<0.01, ***p<0.001.

### 4.2.2 The actin-binding protein Eps8 mimics Wnt3a- and Dvl1-mediated axonal remodelling and F-actin accumulation.

As Eps8 is an actin-binding protein and interacts with Dvl1 (Chapter 3), this protein could mediate the effect of Wnt-mediated axon remodelling, in particular the changes in actin cytoskeleton. To examine this, we first performed gain of function studies and found that Eps8 induces axonal remodelling manifested by a significant increase in growth cone size when compared to control GFP-expressing DRG neurons (Figure 4.3A and 4.3B, cnt: 58; Eps8: 45 growth cones). We also found that Eps8 induces a significant increase in F-actin accumulation (Figure 4.3A and 4.3C). Thus, Eps8 mimics the effect of Wnt3a and Dvl1 gain of function on axonal remodelling.

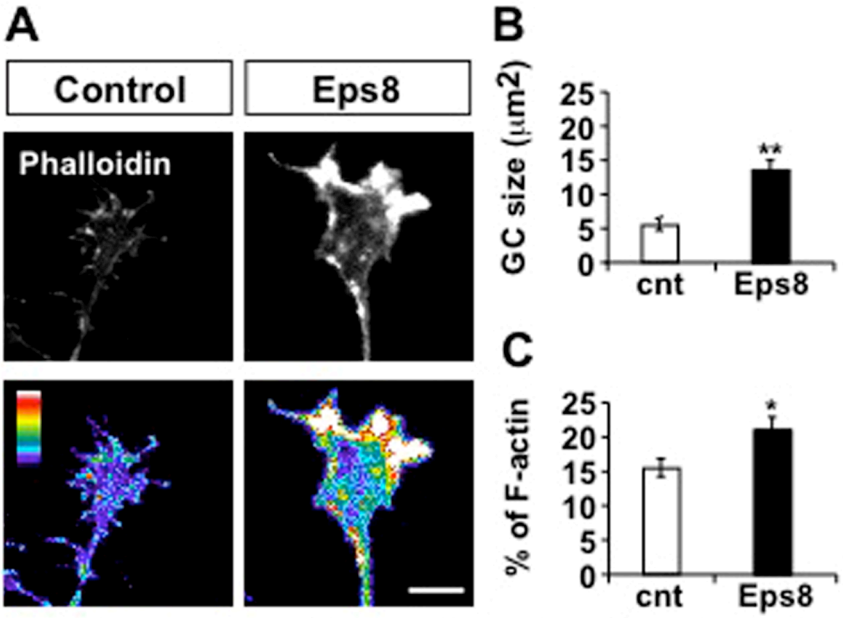



**Figure 4.3: Eps8 induces axonal remodeling and F-actin accumulation in growth cones.** Eps8 promotes axonal remodeling and increases F-actin accumulation in growth cones (A). Pseudocolor images of growth cones labelled with phalloidin highlight the differences in F-actin levels between Eps8 and control GFP-expressing cells. Scale bar: 5μm. Growth cone area is larger in Eps8-expressing growth cones than in controls (B), as is the percentage of growth cone area with bright F-actin fluorescence (C). *p<0.05, **p<0.01.

Eps8 binds directly to actin filaments and induces actin capping and bundling via its C-terminal actin-binding/effector domain (Hertzog et al 2010). In addition, Eps8 activates Rac1 through its SH3 domain (Scita et al 1999, Scita et al 2001), thus regulates the actin cytoskeleton via direct and indirect mechanisms. To

begin to understand the mechanism by which Eps8 regulates axonal remodelling, we examined the effect of a truncated form of Eps8 (Δ533-821; Eps8ΔC) that lacks the SH3 and the actin-binding domain (effector domain) (Figure 4.4A), but still shows an interaction with Dvl1 (Figure 3.8 and 3.9). Interestingly, we found that Eps8ΔC (Δ553-821) also promotes axonal remodelling (Figure 4.4B and 4.4C, cnt: 38; Eps8: 34; Eps8ΔC: 17 growth cones). Thus, the C-terminus of Eps8, which mediates Rac1 activation and direct binding to actin filaments, is not important for axonal remodelling.

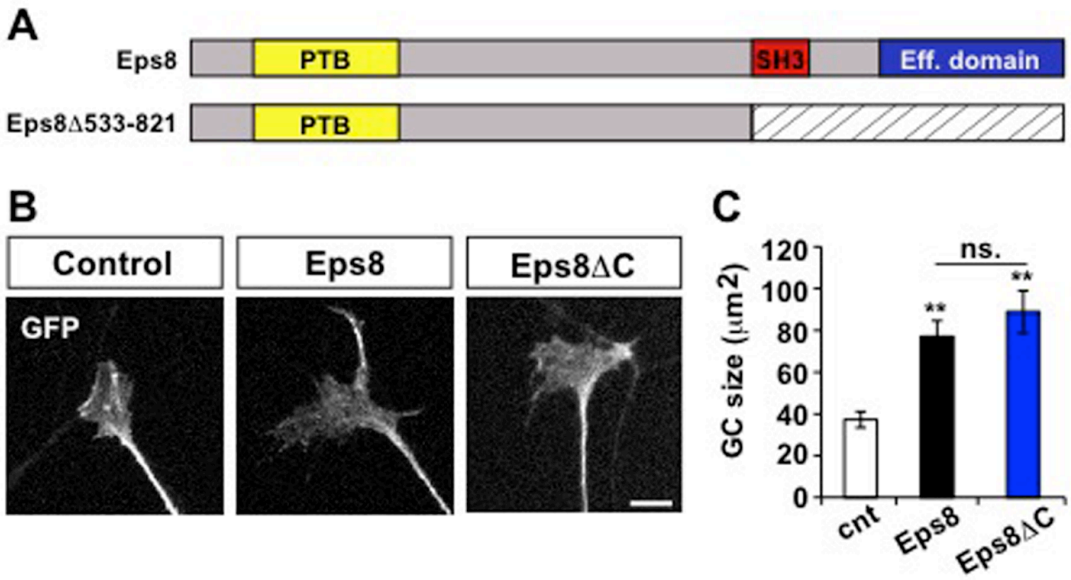



**Figure 4.4: Eps8 induces axonal remodeling through aa 1-533.** (A) Illustration of the Eps8 domains and the constructs used in (B). Expression of a truncated Eps8 that lacks the whole C-terminus (ΔC) induces axonal remodeling similar to the full-length Eps8 (B and C). Scale bar: 10μm. **p<0.01.

### 4.2.3 Eps8 is required for Wnt3a-mediated axonal remodelling.

Eps8 mimics Wnt3a-induced axonal remodelling and accumulation of F-actin. However, it remains unanswered whether Eps8 is a downstream effector of Wnt3a during axonal remodelling. To address this question, we performed loss of function studies using shRNA-mediated Eps8 knockdown (Eps8 KD) (Figure 4.5A). To obtain a significant level of knockdown, DRG neurons were transfected with a combination of three different shRNAs that specifically target Eps8. Neurons expressing scrambled control shRNA construct were used as controls. To first verify that we efficiently knockdown Eps8, we quantified the total intensity of endogenous Eps8 in growth cones and found a significant reduction in Eps8 levels (Figure 4.5B, scr: 13; Eps8 KD: 9 growth cones). In addition, we examined the

number of axonal filopodia, as previous studies have shown that loss of Eps8 results in increased filopodium density in axons (Menna et al 2009). Indeed, we observed that cells expressing shRNAs against Eps8 exhibit a 48% increase in axonal filopodium density (Figure 4.5C and 4.5D, scr: 34; Eps8 KD: 28 cells), demonstrating a significant loss of function effect of Eps8 (Menna et al 2009).

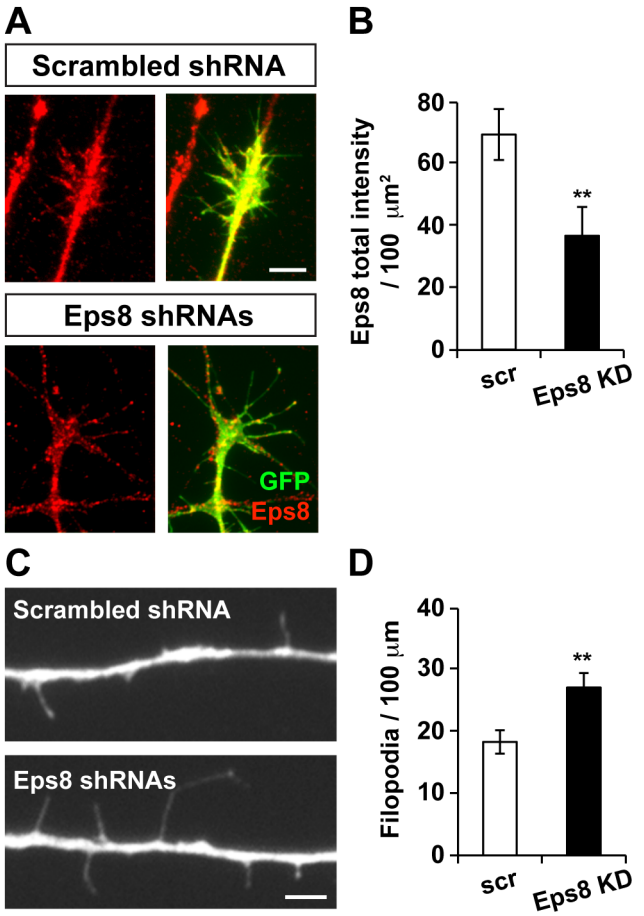



**Figure 4.5: Loss of function of Eps8 induces filopodium formation in axons.** (A) Neurons expressing shRNAs against Eps8 show a significant decrease in the levels of endogenous Eps8. Scale bar: 5 μm. (B) Eps8 KD significantly increases the number of axonal filopodia. Scale bar: 2 μm. **p<0.01.

We then examined the impact of Eps8 loss of function on Wnt3a-induced growth cone remodelling (Figure 4.6A). We found that Wnt3a induces axonal remodelling in control "scrambled" shRNA-expressing cells by increasing both the percentage of cells that showed enlarged growth cones (Figure 4.6B, scr+cnt: 102; scr+Wnt3a: 97; Eps8 KD+cnt: 79; Eps8+Wnt3a: 73 cells) and the

average growth cone area (Figure 4.6C, scr+cnt: 31; scr+Wnt3a: 23; Eps8 KD+cnt: 25; Eps8+Wnt3a: 25 growth cones), as we have previously shown (Purro et al 2008). In contrast, Eps8 shRNA-expressing neurons do not remodel in the presence of Wnt3a (Figure 4.6A-C). These experiments demonstrate that Eps8 is required for Wnt3a signalling to induce axonal remodelling.

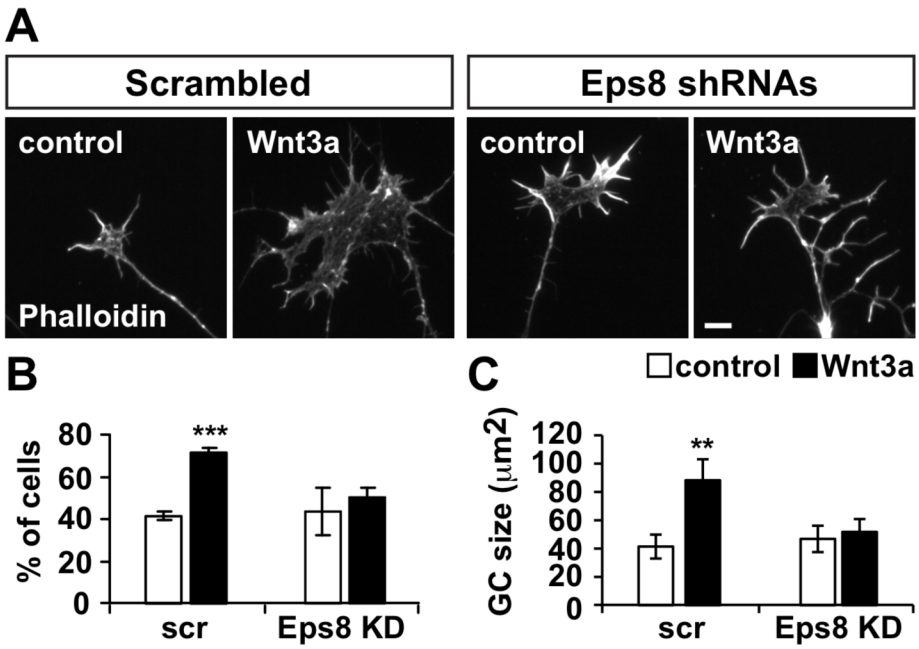



**Figure 4.6: Eps8 knockdown abolishes Wnt3a-induced axonal remodelling.** (A) DRG neurons expressing scramble or Eps8 shRNAs were treated with Wnt3a for 2 hrs. Scale bar: 5μm. (B) In scrambled shRNA-expressing cells Wnt3a increases the percentage of cells that show axonal remodelling and (C) promotes growth cone enlargement. In contrast, neurons expressing Eps8shRNAs do not respond to Wnt3a. **p<0.01, ***p<0.001.

## 4.3 DISCUSSION

Wnt3a induces axonal remodelling through changes in the microtubule and actin cytoskeletons (Hoyos-Flight 2005, Krylova et al 2002, Purro et al 2008). However, the molecular mechanisms induced by Wnt3a to regulate the actin cytoskeleton were not well understood. Here, we show that Wnt3a also promotes profound changes in the actin cytoskeleton during axon remodelling through a pathway that involves the scaffold protein Dvl1, Eps8, an actin-binding protein that directly interacts with Dvl1, and inhibition of Gsk3β. Importantly, Eps8 is required for Wnt3a-mediated axonal remodelling. These findings reveal a novel role for Eps8 in Wnt-mediated axonal remodelling.

Wnts signal through a divergent Wnt canonical pathway to regulate the cytoskeleton during axon remodeling. Our previous studies have shown that Wnt3a through Dvl1 and inhibition of Gsk3β, but not transcription, induces changes in the microtubule cytoskeleton during remodelling (Purro et al 2008). Similarly remodelling at the Drosophila NMJ has shown that Wg promotes microtubule reorganization at synaptic boutons through a pathway that requires Shaggy/Gsk3, but independently of transcription (Miech et al 2008). Here we showed that Wnt3a signalling through Dvl1 and inhibition of Gsk3β is also involved in regulating the actin cytoskeleton. Thus, activation of a Wnt divergent canonical pathway is responsible for both actin and microtubule cytoskeletal changes during axon terminal remodelling.

Actin-binding proteins play a crucial role in the regulation of actin dynamics and organisation. Among the actin binding proteins is Eps8, a multifunctional protein that regulates the actin cytoskeleton through direct and indirect mechanisms (Disanza et al 2004, Disanza et al 2006, Scita et al 1999, Scita et al 2001). We found that Eps8, which directly interacts with Dvl1, promoted axonal remodelling and accumulation of F-actin in growth cones. How does Eps8 induce axonal remodelling? Previous studies showed that Eps8 acts as a capping protein to regulate filopodium and spine formation in hippocampal neurons (Menna et al 2009, Menna et al 2013, Stamatakou et al 2013). Consistent with these findings, we found that loss of function of Eps8 increased axonal filopodium density in DRG neurons. These results suggest that Eps8 promotes axonal remodelling through its actin capping activity. However, high capping activity would lead to a decrease in F-actin content (Disanza et al 2004, Stamatakou et al 2013, Van Impe et al 2013) rather than an increase as we found in our Eps8 gain of function studies. Moreover, deletion of the whole C-terminus of Eps8 (Eps8Δ533-821) does not impair its ability to induce remodelling, indicating that neither direct binding to actin nor Rac1 activation are required in this process.

How does Eps8 contribute to Wnt-mediated remodelling? Eps8 has been shown to activate Akt, a kinase that phosphorylates and inactivates Gsk3β (Cohen & Frame 2001, Woodgett 2005). Indeed, Eps8 enhances cell proliferation and migration through the PI3K-Akt pathway and increases β-catenin levels (Sala & Segal 2014, Wang et al 2009, Wang et al 2010). Importantly, expression of a

constitutively active form of Akt in DRG neurons increases branching and growth cone size (Grider et al 2009), mimicking the effects observed upon activation of the Wnt3a-Dvl1-Gsk3β pathway (Purro et al 2008). Given that Eps8 interacts with Dvl1, which is upstream of Gsk3β, our findings raise the possibility that Wnt3a regulates axonal remodelling through a pathway where interaction of Eps8 with Dvl1 activates Akt, leading to Gsk3β inhibition and resulting in growth cone enlargement and axon remodelling.

In summary, our studies demonstrate that Wnt signalling acts through Dvl1 and Gsk3β, to directly induce changes in the actin cytoskeleton in axonal growth cones. Wnt3a-induced actin accumulation and axonal remodelling is mediated by the actin-binding protein Eps8 through its direct interaction with Dvl1. Importantly, loss of function of Eps8 blocks the ability of Wnt3a to induce axonal remodelling. These data provide important insights into the mechanisms that are induced by Wnt signalling during axonal remodelling and demonstrate a novel role for Eps8. In the next chapter we aim to test the role of Eps8 in synapse formation and Wnt7a-mediated spine morphogenesis.

# Chapter 5:

# The role of Eps8 in synaptogenesis and Wnt-mediated spine growth

# 5.1 INTRODUCTION

The formation and growth of spines, actin-rich dendritic protrusions that receive excitatory input, is crucial for the assembly of functional neuronal circuits. Increased spine density correlates with an increased number of excitatory synapses, whereas spine growth is associated with changes in synaptic strength (Bourne & Harris 2008, Segal 2010, von Bohlen Und Halbach 2009). Dendritic spine morphogenesis is critically dependent on actin dynamics, a process that is modulated by signalling molecules (Schubert & Dotti 2007, Tada & Sheng 2006), through poorly defined mechanisms.

Wnt secreted factors regulate various aspects of neuronal development, from axonal and dendritic outgrowth to synapse formation and maintenance (Budnik & Salinas 2011, Park & Shen 2012, Rosso & Inestrosa 2013). Recent studies have demonstrated that Wnts promote spine formation and growth (Ciani et al 2011, Farias et al 2009, Hiester et al 2013, Sharma et al 2013, Varela-Nallar et al 2010). Importantly, postsynaptic activation of the Wnt signalling pathway, by expressing Dishevelled 1 (Dvl1) induces spine growth through a pathway that involves local CaMKII activation (Ciani et al 2011). The requirement of Wnt7a signalling in spine morphogenesis has been also showed *in vivo*, since the Wnt7a; Dvl1 double knock out mice exhibit defects in spinogenesis (Ciani et al 2011). These findings clearly demonstrate a crucial role of Wnt proteins at the postsynaptic side in addition with their previously reported role in presynaptic assembly. However, the downstream target(s) of Wnts during spine formation and growth remain elusive.

Several actin-binding proteins have been shown to affect spine formation and morphology (Table 1.1). Eps8 is an actin-binding protein that was identified as a direct interactor of Dvl1 in the brain (Chapter 3). In neurons, Eps8 is prominently enriched in the axonal growth cone, where it inhibits filopodium formation through its capping activity (Menna et al 2009, Vaggi et al 2011). Although Eps8 localizes to both the presynaptic and postsynaptic sides (Offenhauser et al 2006, Proepper et al 2007, Sekerkova et al 2007), its role in synapse formation has not been examined.

The aim of this chapter was to examine whether Eps8 is a downstream effector of Wnt signalling during spine growth. We show that Eps8 is enriched in the PSD fraction of synaptosomal preparations and is localized in spine heads. Gain of function studies demonstrate that Eps8 promotes spine formation and alterations in spine morphology. In addition, we show that Eps8 promotes the localization of excitatory synapses on dendritic spines. However, Eps8 does not affect spine growth neither modulates Dvl1-mediated spine effects. These results show that Eps8 regulates the formation and maturation of dendritic spines through a Wnt-independent pathway.

# 5.2 RESULTS

## 5.2.1 Eps8 is enriched in the postsynaptic compartment and localises into dendritic spines.

To determine the potential role of Eps8 in synapse formation, we first examined its localization at central synapses. We found that Eps8 is present in both pre- and post- synaptosomal fractions (Figure 5.1A), as previously reported (Offenhauser et al 2006, Proepper et al 2007). However, Eps8 is primarily enriched in the postsynaptic density (PSD) when compared with the presynaptic fraction (synaptosomal membrane fraction - SMF) (Figure 5.1A). To analyse in more detail the localization of endogenous Eps8 within the dendritic compartment, we used hippocampal neurons expressing EGFP-actin, which allows the easy identification of dendritic spines. Consistent with the biochemical results, we observed that endogenous Eps8 is prominent in dendritic spines and is particularly enriched at spine heads (Figure 5.1B). Together these results demonstrate that Eps8 is enriched at dendritic spines.

## 5.2.2 Eps8 promotes the formation of dendritic spines.

The localisation of Eps8 on dendritic spines prompted us to examine its potential role in dendritic spine morphogenesis. Gain of function studies in hippocampal cultures, where Eps8 was expressed together with EGFP-actin (Figure 5.1C), reveals that Eps8 induces a 63% increase in spine density (Figure 5.1D) with a concomitant decrease in filopodium density (Figure 5.1E, cnt: 37; Eps8 40 cells). Although Eps8 does not affect spine size (Figure 5.1F), it does change the

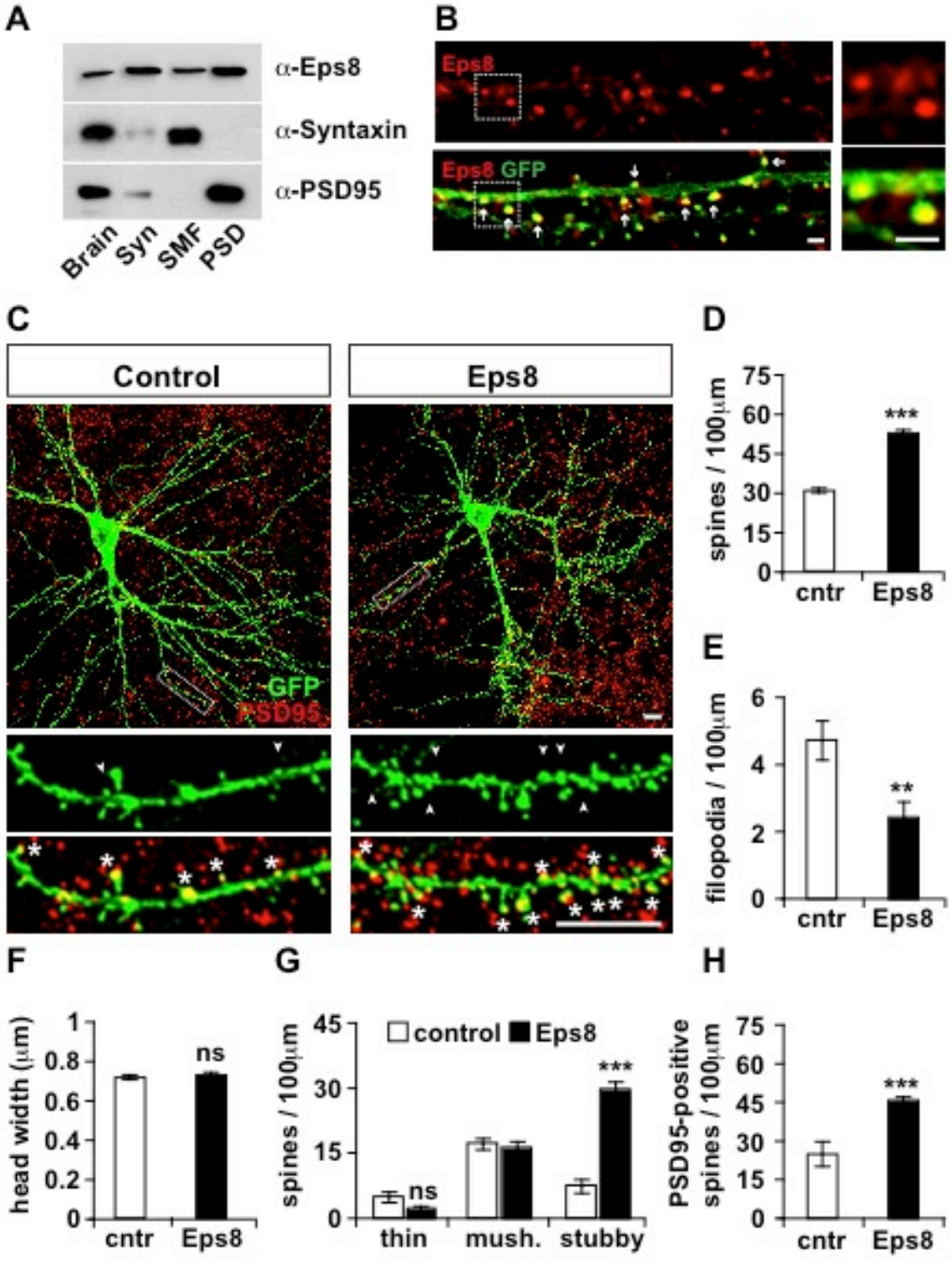



**Figure 5.1: Eps8 is localized at synapses and promotes spine formation.** (A) Eps8 is enriched in synaptosomes isolated from adult mouse brains and specifically in the PSD fraction. Brain: brain homogenate, Syn: synaptosomes, SMF: synaptosomal membrane fraction (presynaptic and soluble postsynaptic fraction), PSD: postsynaptic density. (B) Endogenous Eps8 localizes into dendritic spines (arrows). Scale bars: 2μm. (C) Hippocampal neurons expressing Eps8 exhibit increased spine density. Scale bars: 10μm. Quantification shows that Eps8 increases the number of spines (D), whereas it decreases filopodium density (E), while spine size is unaffected (F). Eps8 specifically increases the number of stubby spines (arrowheads; G) and the number of spines containing PSD95 (asterisks; H). **p<0.01, ***p<0.001, ns: non-significant.

morphology of spines, as the number of stubby spines is significantly increased without affecting the number of thin and mushroom spines (Figure 5.1G, cnt: 18; Eps8: 20 cells). Similar results were obtained in neurons expressing Eps8 and EGFP (data not shown, cnt: 10; Eps8: 10 cells); we therefore continued our studies using EGFP-actin, which labels more efficiently dendritic spines. In addition, we found that Eps8 increases the number of spines containing PSD95 (Figure 5.1H, cnt: 18; Eps8: 20 cells), a postsynaptic marker that accumulates within spine head during spine maturation (Han & Kim 2008, McMahon & Diaz 2011). These results demonstrate that Eps8 induces the formation and maturation of dendritic spines. However, Eps8 effect is distinct to that of Dvl1, which affects spine growth without affecting spine number, suggesting that Eps8 does not have a functional interaction with Dvl1 during spine morphogenesis.

### 5.2.3 Eps8 regulates the balance between spine and shaft excitatory synapses.

To gain more insight into the role of Eps8 in synapse formation, we examined whether Eps8 affects synapse density. Excitatory synapses were defined by the apposition of the presynaptic marker vGlut1 to the postsynaptic NMDA receptor subunit GluN1 (Figure 5.2A). We found that Eps8 gain of function did not affect the total density of excitatory synapses (synapses on spines and on dendritic shafts) (Figure 5.2B, cnt: 19; Eps8: 17 cells). Given that Eps8 increases spine density (Figure 5.1), these results could suggest that Eps8 augments the proportion of excitatory synapses on spines. Indeed, we found that Eps8 increases the number of synapses on spines, whereas it decreases the number of shaft synapses (Figure 5.2C and 5.2D). These results demonstrate that Eps8 increases the number of innervated spines at the expense of shaft synapses.

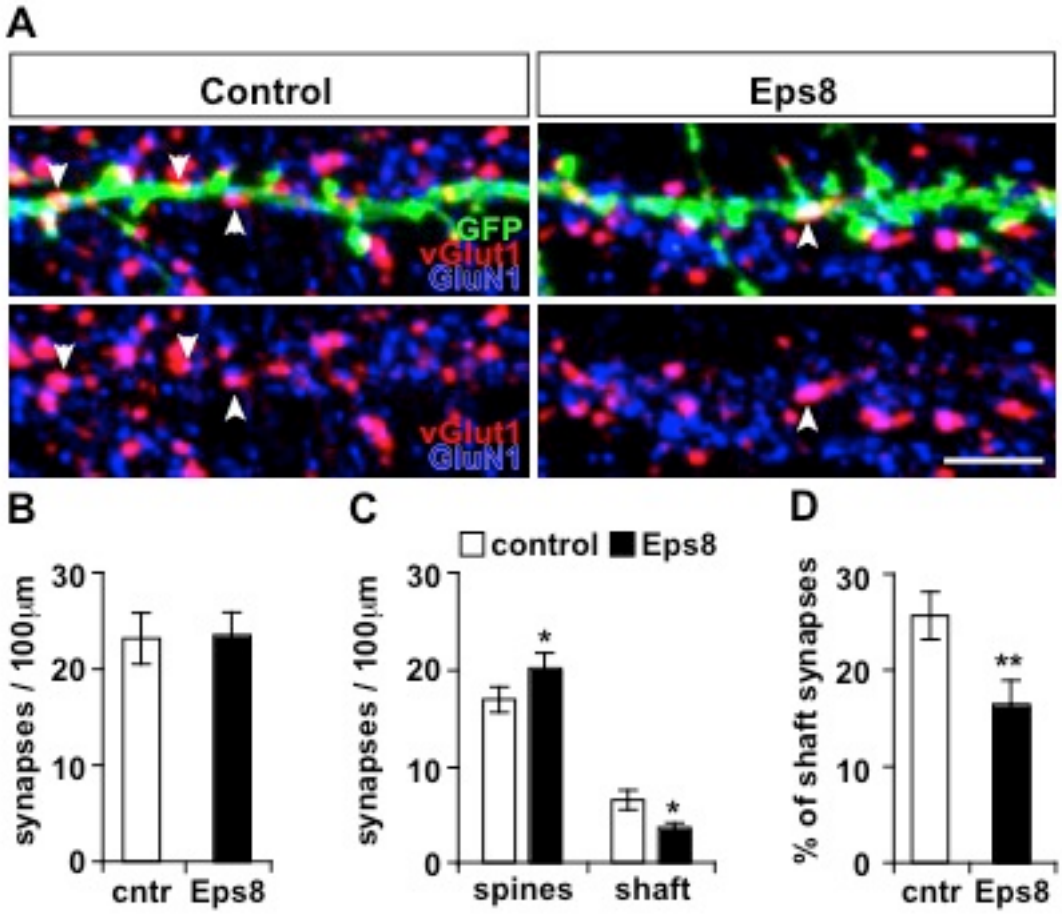



**Figure 5.2: Eps8 induces a shift towards excitatory synapses on spines.** Analysis of synapse density (apposition of vGlut1 to GluN1 puncta) (A) reveals that Eps8 expression does not affect total synapse density (B) but increases the number of synapses on spines and decreases the number of shaft synapses (A, arrowheads; C, D). Scale bar, 5 µm. *p < 0.05. **p < 0.01.

### 5.2.4 Eps8 induces spine formation through its C-terminus.

To begin to understand the mechanism by which Eps8 regulates spine morphogenesis, we first examined the effect of the truncated form of Eps8 (Δ533-821, Eps8ΔC) that lacks the SH3 and the actin-binding domain (effector domain) (Figure 5.3A), but still shows an interaction with Dvl1 (Figure 3.8 and 3.9). The SH3 domain is required for interaction with Abi1 (Scita et al 1999), whereas the effector domain binds directly to actin filaments and induces actin capping and bundling (Hertzog et al 2010). In contrast to the full-length Eps8, Eps8Δ533-821 does not affect spine formation (Figure 5.3B-D). These results demonstrate that Eps8 promotes spine formation through its C-terminus domain

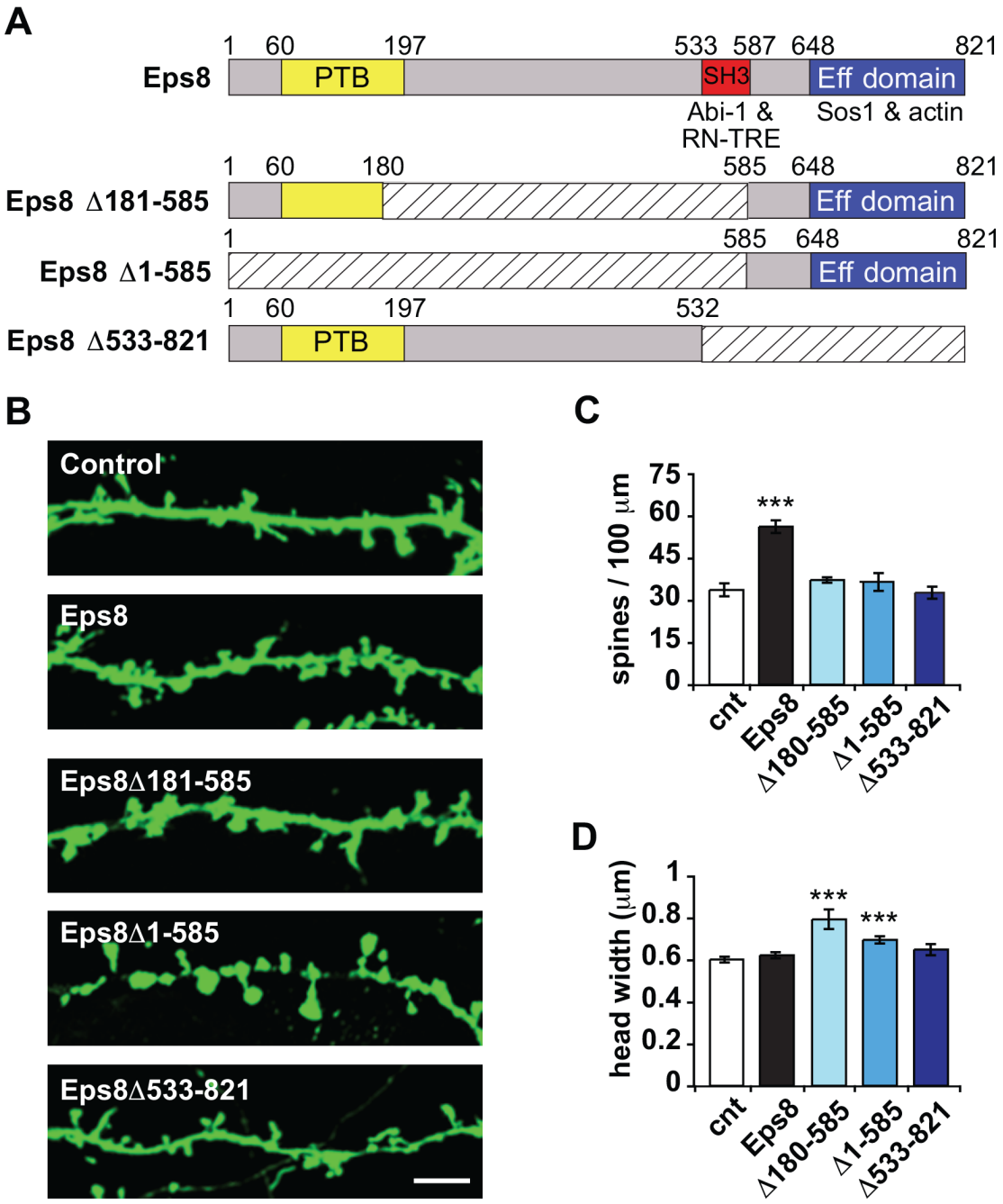



**Figure 5.3: The C-terminus of Eps8 is required for induction of spine number.** (A) Eps8 deletions constructs tested for their effect on spine morphogenesis. (B) Hippocampal neurons were transfected with GFP-actin to visualize spines and with full-length Eps8 or with the different Eps8 deletion mutants. Scale bar: 5μm. (C and D) Expression of the truncation construct lacking the whole C-terminus (Δ533-821) has no effect in spine formation and growth. Conversely the deletions that possess the effector domain (648-821 aa) show an increase in spine growth. ***p < 0.001.

containing the actin-binding domain, suggesting that Eps8 modulates spine morphogenesis through changes in the actin cytoskeleton.

To further examine the function of Eps8 in spine formation, we expressed another two truncated versions of Eps8 - Δ1-585 and Δ181-585 (Figure 5.3A). We found that both truncated forms do not affect spine number, but promote spine growth (Figure 5.3B-D), an effect that full-length Eps8 does not show. We hypothesize this effect on spine growth is mediated by the actin-binding domain of Eps8 (effector domain: 647-821aa), which possesses a barbed-end capping and bundling activity. However, further analysis is required to fully understand the role of Eps8 in spine formation and growth.

### 5.2.5 Eps8 does not affect Dvl1-mediated spine growth.

As mentioned before, Dvl1 expression results in increase spine head size, without affecting spine density (Ciani et al 2011). To examine whether Eps8 can modulate the Dvl1-mediated effect in spine growth, we co-expressed both proteins to determine their combinatorial effect during spinogenesis (Figure 5.4A). Neurons transfected with both Eps8 and Dvl1 have more and bigger spines (Figure 5.4B and 5.4C, cnt: 17; Eps8: 18 cells; Dvl: 15; Eps8+Dvl1: 18 cells), an effect that seems to be a result of Eps8 and Dvl1 independent actions, respectively. To further examine a possible functional link between Dvl1 and Eps8 we co-expressed Dvl1 and the Eps8Δ533-821 truncation (Figure 5.5A), a deletion mutant that still interacts with Dvl1, but has no effect in spine number or head width (Figure 5.3C and 5.3D). Expression of the Eps8Δ533-821 has no effect on Dvl1 function regarding spine size, as Dvl1 is still able to increase spine head size, without affecting spine density (Figure 5.5B and 5.5C, cnt: 12; Dvl: 8; Δ533-821+Dvl1: 10 cells). We conclude that Eps8 does not synergise with Dvl1 to induce spine growth.

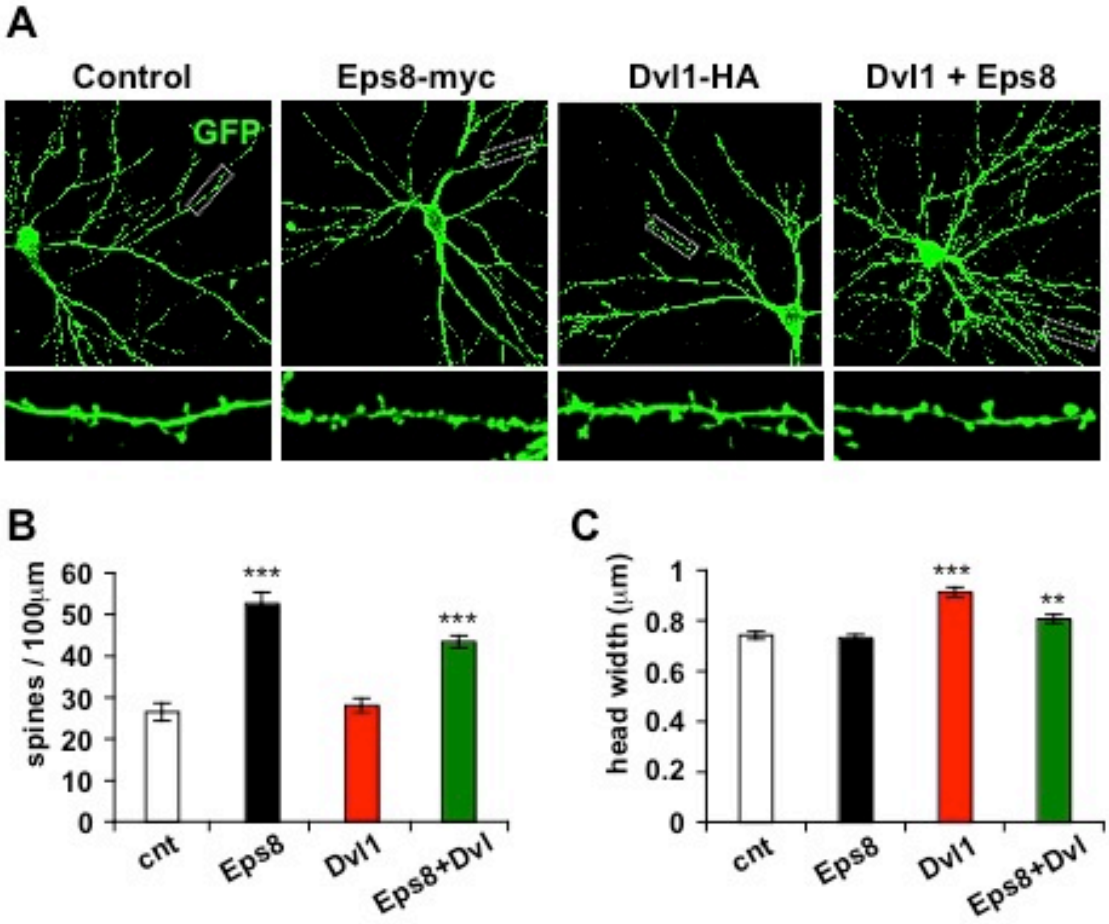



**Figure 5.4: Eps8 does not affect the function of Dvl1 on spine growth.** (A) Hippocampal neurons were co-transfected with GFP-actin, Eps8 and/or Dvl1. Scale bars: 10μm. Quantification shows that Eps8 and Dvl1 co-expression has a complementary effect in spine morphogenesis with more (B) and bigger spines (C). **p<0.01, ***p<0.001

## 5.3 DISCUSSION

The formation and maturation of dendritic spines is highly-dependent on actin dynamics, a process that is tightly regulated by secreted and membrane-tethered proteins (Penzes & Cahill 2012, Schubert & Dotti 2007, Tada & Sheng 2006). Previous results from our lab have demonstrated that Wnt signalling induces spine growth through Dvl1 and CaMKII (Ciani et al 2011). In addition, we have found that Eps8, an actin regulating protein, is a direct interactor of Dvl1 in the brain (Chapter 3). The aim of this chapter was to examine whether Eps8 acts downstream of the Wnt signalling to induce spine growth. We show that Eps8 is enriched in spine heads and induces spine morphogenesis through its C-terminus domain. However, this effect is probably through a pathway that does not involve Dvl1, since Eps8 did not affect Dvl1-mediated spine growth.

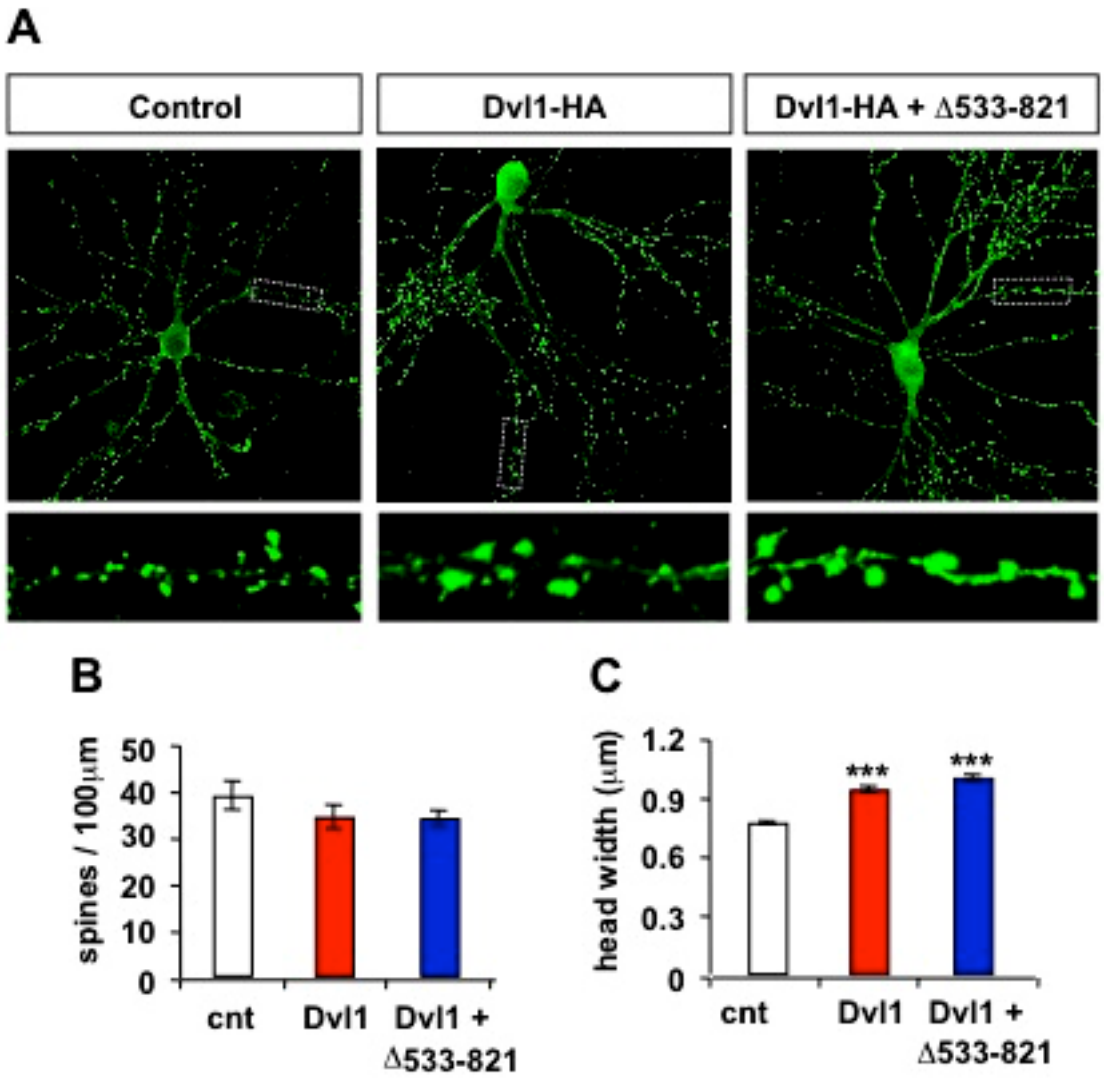



**Figure 5.5: Eps8Δ533-821 does not affect the function of Dvl1 on spine size.** (A) Neurons were co-transfected with GFP-actin, Dvl1 and Eps8Δ533-821. Scale bars: 10μm. (B and C) Quantification shows that Eps8Δ533-821 does not modulate Dvl1 effect in spine size. ***p<0.001

The most accepted model for spine development starts with the initiation of a dendritic filopodium, which through the combinatorial action of several actin-binding proteins, elongates and then converts into an immature spine with the formation of a spine head on its tip (Ethell & Pasquale 2005, Yuste & Bonhoeffer 2004). Our results show that gain of function of Eps8 results in more spines, with the concomitant decrease in filopodium density. In addition we show that Eps8 promotes spine maturation, since there is also an increase in spines positive for the postsynaptic scaffold protein PSD95. These findings demonstrate that Eps8 regulates the formation or stabilisation of mature spines.

Another model for spine development describes the emerge of stubby spines from shaft synapses, which are mainly present in young neurons (Boyer et al 1998, Fiala et al 1998, Miller & Peters 1981, Reilly et al 2011). However, very little is known about the mechanisms that regulate the formation of spine versus shaft synapses. Here, we show that Eps8 induces the formation of stubby spines without affecting the number of thin or mushroom type spines. In addition, we found that Eps8 promotes the formation of excitatory synapses on spines with a concomitant decrease in the number of excitatory shaft synapses. Our results suggest that Eps8 may induce the *de novo* formation of dendritic spines with stubby morphology in sites where shaft synapses were present. However, further experiments are required to address this hypothesis. Importantly, these data provide important insights into the mechanisms that regulate the localization of excitatory synapses on dendritic spines.

Postsynaptic Wnt7a signalling induces dendritic spine growth and synaptic strength (Ciani et al 2011). This effect is mediated by Dvl1 and local CaMKII activation (Ciani et al 2011). We found that co-expression of Eps8 and Dvl1 induces both spine number and growth. In addition, expression of an Eps8 deletion mutant that lacks the C-terminus, but still interacts with Dvl1, had no effect in Dvl1-mediated spine growth. These data indicate that Eps8 acts independently of Dvl1 to induce spine formation.

In summary, our data demonstrate that Eps8 stimulates the formation of dendritic spines, through its C-terminus, without affecting their size. Interestingly, Eps8 induces a shift of shaft excitatory synapses towards synapses on spines. These data provide important insights into the mechanisms of excitatory synapse localisation on spines. In addition, our findings show that Eps8 is probably not involved in Wnt-mediated responses during spine growth. In the following and last result chapter we will further explore the role of Eps8 in spine morphogenesis and in particular its contribution in activity-mediated structural and functional plasticity.

# Chapter 6:

# Activity-dependent spine morphogenesis: a role for the actin-capping protein Eps8

# 6.1 INTRODUCTION

Dendritic spine morphogenesis is critically dependent on actin dynamics, a process that is modulated by signalling molecules (Schubert & Dotti 2007, Tada & Sheng 2006) and neuronal activity (Bosch & Hayashi 2011, Hotulainen & Hoogenraad 2010). Indeed, long-term potentiation (LTP) promotes the formation and growth of spines through changes in actin dynamics (Bosch & Hayashi 2011, Bramham 2008, Carlisle & Kennedy 2005, Cingolani & Goda 2008). Interestingly, changes in the actin cytoskeleton are required for the maintenance of LTP (late LTP), but not for its induction (early LTP) (Fukazawa et al 2003, Krucker et al 2000, Ramachandran & Frey 2009). However, the molecular mechanisms by which neuronal activity regulates spine morphogenesis through changes in the actin cytoskeleton remain poorly understood.

Actin-capping proteins play a key role in the assembly and elongation of actin filaments. Eps8 is a multi-functional protein that regulates the actin cytoskeleton (Disanza et al 2004, Disanza et al 2006, Offenhauser et al 2006, Scita et al 1999). We previously demonstrated that Eps8 is enriched at dendritic spine heads and induces spine morphogenesis in a Wnt-independent manner (Chapter 5). The aim of this chapter is to examine in detail the role of Eps8 in spine formation and particularly in activity-mediated structural and functional plasticity. Using loss of function studies we demonstrate that Eps8 is required for the formation of dendritic spines, whereas inhibits filopodium formation. Eps8 knockdown increases actin polymerization and induces fast actin turnover within dendritic spines, as revealed by free-barbed end and FRAP assays, consistent with a role for Eps8 as an actin-capping protein. Interestingly, Eps8 loss of function induces a shift towards excitatory synapses on the dendritic shaft. Importantly, Eps8 loss of function impairs the structural and functional plasticity of synapses induced by long-term potentiation. These findings demonstrate a novel role for Eps8 in spine formation and in activity-mediated synaptic plasticity.

# 6.2 RESULTS

## 6.2.1 Eps8 knockdown decreases spine formation and affects spine morphology.

To further investigate the role of Eps8 in spine morphogenesis, we examined the consequence of Eps8 loss of function by performing shRNA-mediated knockdown (KD) experiments. We used a combination of three shRNAs to obtain a significant level of knockdown, as measured by the level of endogenous Eps8 protein in the cell soma and dendritic processes of shRNA-expressing cells (Figure 6.1A). We observed a 79% reduction in the total intensity level of Eps8 in the cell soma (Figure 6.1B, scr: 23; Eps8 KD: 20 cells) and a 69% decrease in the Eps8 puncta density on dendrites (Figure 6.1C) in neurons expressing Eps8 shRNAs when compared to neurons expressing the scrambled shRNA. Thus, Eps8 shRNAs effectively down-regulate the levels of endogenous Eps8 protein in cultured hippocampal neurons.

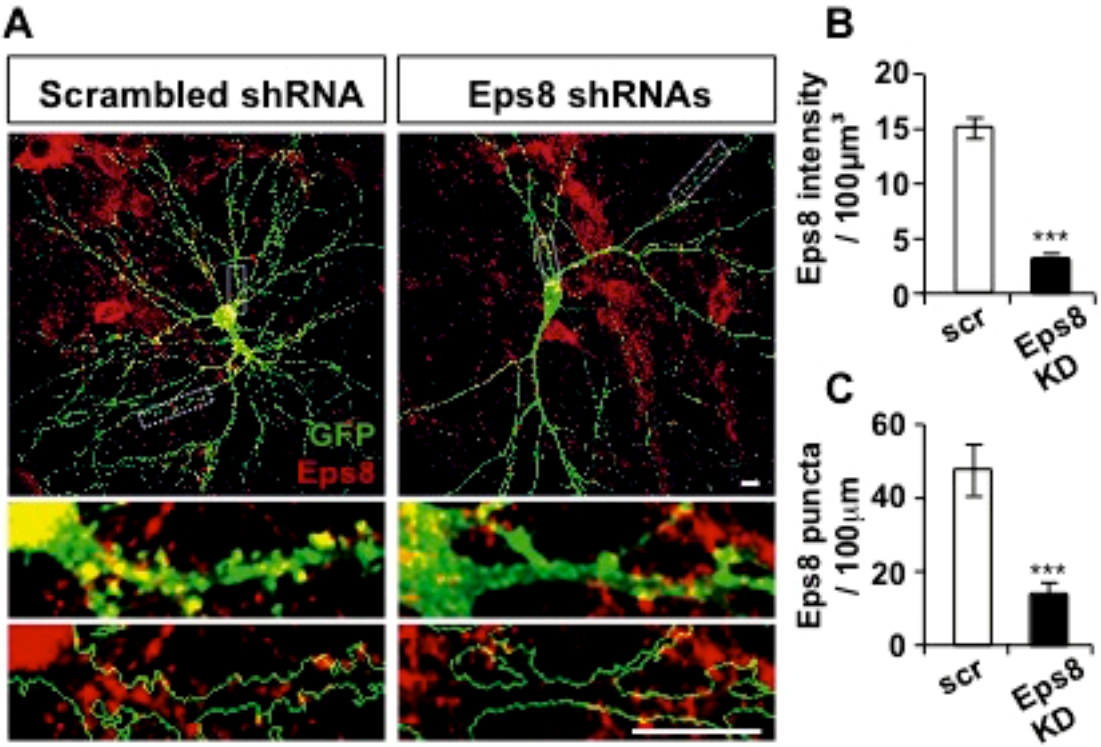



**Figure 6.1: Eps8 knockdown using shRNAs.** (A, B, and C) shRNA-mediated knockdown of Eps8 significantly decreases the total intensity level of endogenous Eps8 normalized to soma volume and Eps8 puncta density on dendrites. Scale bars: 10μm. **p<0.01, ***p<0.001.

We next examined the impact of Eps8 loss of function on spine morphogenesis (Figure 6.2A). Expression of Eps8 shRNAs decreases spine density by 39% (Figure 6.2B, scr: 32; Eps8 KD: 40 cells), whereas significantly increases filopodium density by 46% (Figure 6.2C). In addition, Eps8 shRNAs-expressing cells have fewer small spines with a concomitant increase in the number of large spines (Figure 6.2D, scr: 20; Eps8 KD: 20 cells) resulting in a significant increase in the average spine size (Figure 6.2E). We confirmed the specificity of the Eps8 KD, as we found that the three different shRNAs independently decrease spine density (scrambled: 32 ± 1.63, 15 cells; shRNA#1: 21 ± 1.61, 18 cells; shRNA#2: 19 ± 1.59, 16 cells; shRNA#3: 21 ± 0.92 spines per 100 mm dendritic length, 16 cells), while increasing spine size (scrambled: 0.66 ± 0.03 mm; shRNA#1: 0.77 ± 0.02 mm; shRNA#2: 0.75 ± 0.02 mm; shRNA#3: 0.74 ± 0.02 mm head width). In addition, Eps8 KD induces an increase in the percentage of spines with filopodia growing from spine heads and with irregular spine head shape (Figure 6.2F and 6.2G). These results demonstrate that Eps8 is required for spine formation and regulates spine morphology.

### 6.2.2 Eps8 regulates actin polymerization and turnover within dendritic spines.

To determine whether the capping activity of Eps8 mediates spine formation, we generated a mutant Eps8 (Eps8TM) carrying three single point mutations in the actin-binding domain (V729A, T731A and W732A; Figure 6.2A). This triple mutant retains the actin bundling activity, but is defective in actin capping (Menna et al 2009). We found that Eps8TM decreases spine density by 42%, whereas increases spine growth by 21% (Figure 6.3B and 6.3C, cnt: 14; Eps8: 11; Eps8TM: 17 cells), similarly to the phenotype conferred by Eps8 KD (Figure 6.3E). These results demonstrate that the capping activity of Eps8 is required for spine formation.

To further elucidate the function of Eps8 in spine formation, we examined whether loss of function of Eps8 affects actin dynamics within dendritic spines. To determine the sites of actin polymerization, we performed free barbed-end assays in neurons expressing Eps8 shRNAs (Figure 6.3E). The free (uncapped) actin barbed-ends were visualized by the incorporation of purified G-actin into actin filaments. We found that in control scrambled shRNA-expressing neurons almost 20% of the spines have free-barbed ends (Figure 6.3F), as previously

reported (Gu et al 2010). In contrast, Eps8 KD leads to a significant increase in the percentage of spines that incorporated fluorescent G-actin (Figure 6.3F), thus reflecting an increase in the number of uncapped barbed-ends in spines.

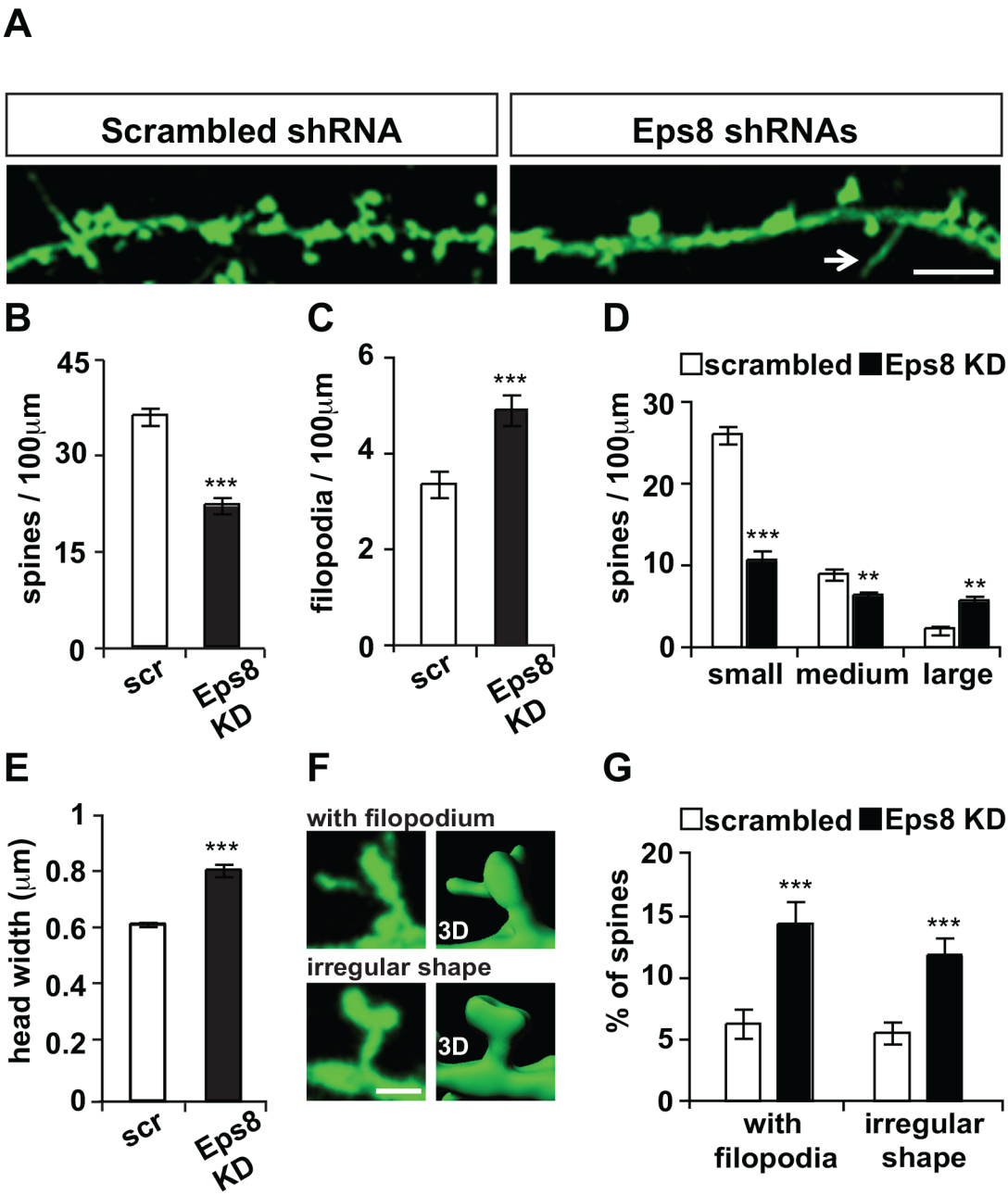



**Figure 6.2: Loss of function of Eps8 decreases the formation of dendritic spines.** Eps8 loss of function (A) decreases spine number (B) and increases filopodium density (C; arrow) Scale bar: 5μm. Distribution of spines according to their size (D) reveals that loss of function of Eps8 decreases the density of small and medium-sized spines with a concomitant increase in the density of large spines, thus increasing the average spine size (E). Spine head width: small ≤ 0.7 μm, medium > 0.7 μm & ≤ 1.0 μm, large > 1.0 μm. The percentage of spines with filopodial protrusions on their head or with abnormal head shape (F – left: fluorescence; right: 3D reconstruction) is increased (G). Scale bar: 2μm. **p<0.01, ***p<0.001.

To further study the role of Eps8 on actin dynamics, we performed FRAP assays (Figure 6.3G) to monitor the turnover of actin filaments at single spines. This assay is based on the fluorescent recovery of EGFP-actin after photobleaching using time-lapse imaging (Koskinen et al 2012, Star et al 2002). Spines from scrambled shRNA-expressing neurons have a half-time fluorescence recovery (t1/2) of 22 s, similar to those obtained in previous studies (Koskinen et al 2012, Star et al 2002). In contrast, spines from Eps8 KD neurons exhibit a faster EGFP-actin fluorescence recovery with $t_{1/2}$ equal to 12 s (Figure 6.3H; scr: 24; Eps8 KD: 31 spines). Thus, the first-order rate constant for the recovery curve of the control scrambled-expressing cells is 0.031 $s^{-1}$, whereas for the Eps8 shRNAs-expressing cells is 0.058 $s^{-1}$. Together, our free barbed-end and FRAP assays demonstrate that Eps8 silencing increases actin polymerization and fast actin turnover, suggesting that Eps8 acts as a capping protein in dendritic spines.

### 6.2.3 Eps8 regulates the balance between spine and shaft excitatory synapses.

To gain more insight into the role of Eps8 in synapse formation we investigated the effect of Eps8 KD on excitatory synapse formation. We found that Eps8 KD does not affect the total density of excitatory synapses (Figure 6.4A; scr: 28; Eps8 KD: 30 cells), like the gain of function of Eps8 (Chapter 5). However, Eps8 KD induces a decrease in the number of synapses on spines with a concomitant increase in the number of shaft excitatory synapses (Figure 6.4B and 6.4C). Therefore, Eps8 KD decreases the number of dendritic spines, but the total number of excitatory synapses remains unchanged due to a shift towards shaft excitatory synapse formation. These loss of function studies demonstrate that Eps8 is required for the proper localisation of excitatory synapses on dendritic spines.

Since Eps8 KD increases the size of dendritic spines, which can be influenced by the content of AMPARs (Matsuzaki et al 2001), we examined the effect of Eps8 KD on the localization of the surface GluA1 AMPAR subunit (sGluA1) at synapses. Synapses were identified by the apposition of vGlut1 to sGluA1 (Figure 6.5A). We found that Eps8 loss of function did not affect the total number of synapses containing sGluA1 along the dendrites (Figure 6.5B, scr: 21; Eps8 KD: 24 cells).

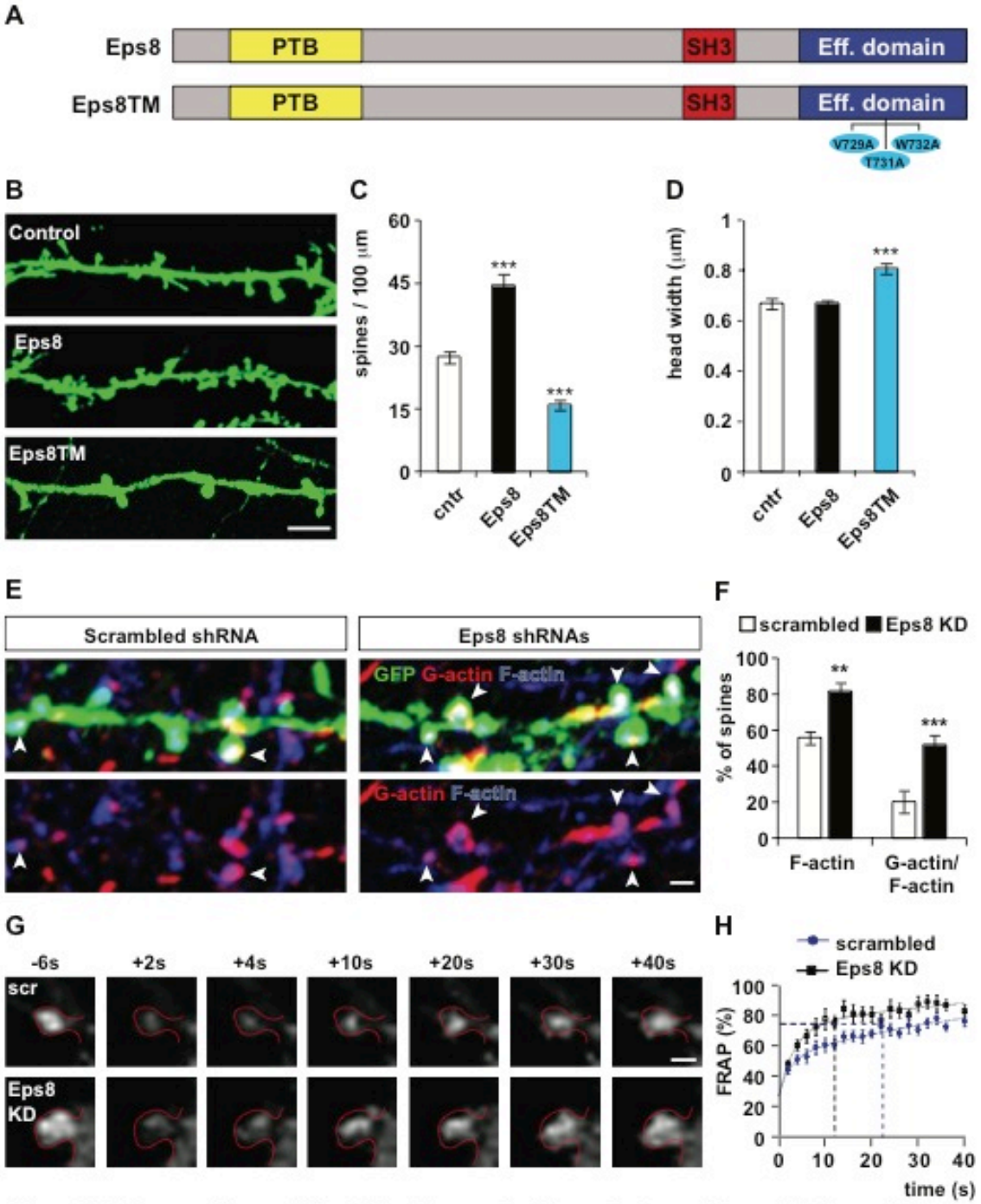



**Figure 6.3: The capping activity of Eps8 is required for spine formation and its loss of function induces actin polymerization and fast actin turnover.** (A) Illustration of the Eps8 domains and the constructs used in (B). Expression of a triple mutant (Eps8TM), that lacks a capping activity, decreases spine density (C) and increases spine growth (D). Scale bar: 5μm. Free actin barbed-ends were visualized with rhodamine-conjugated G-actin. F-actin was labelled with phalloidin (E). Scale bar: 1μm. Analysis of the sites of active actin polymerization within dendritic spines revealed that Eps8 KD leads to an increase in the percentage of spines that have F-actin and free barbed-ends (F). (G) FRAP assays were performed by recording spines expressing EGFP-actin from control scramble shRNA and shRNA Eps8 expressing neurons. EGFP-actin was bleached on single spines and fluorescence recovery was followed by time-lapse microscopy. The frames before (-6s) and after (from +2 to +40s) bleaching are shown. Scale bar: 1μm. (H) Eps8 KD shows a faster recovery rate than control scrambled. Dotted lines represent the $t_{1/2}$ recovery for scrambled (22s) and Eps8 KD (12s). In spines of scrambled-expressing neurons, the fluorescence recovery of EGFP-actin was complete by 110s, whereas in Eps8 shRNAs-expressing neurons complete recovery was achieved within 80s. *p<0.05, **p<0.01, ***p<0.001.

Interestingly, Eps8 silencing increases the number of sGluA1 labelled synapses on the shaft with the concomitant decrease in the number of sGluA1 labelled synapses on spines (Figure 6.5C and 6.5D). Thus, Eps8 induces a shift in the localization of sGluA1-contaning synapses from spines to the dendritic shaft.

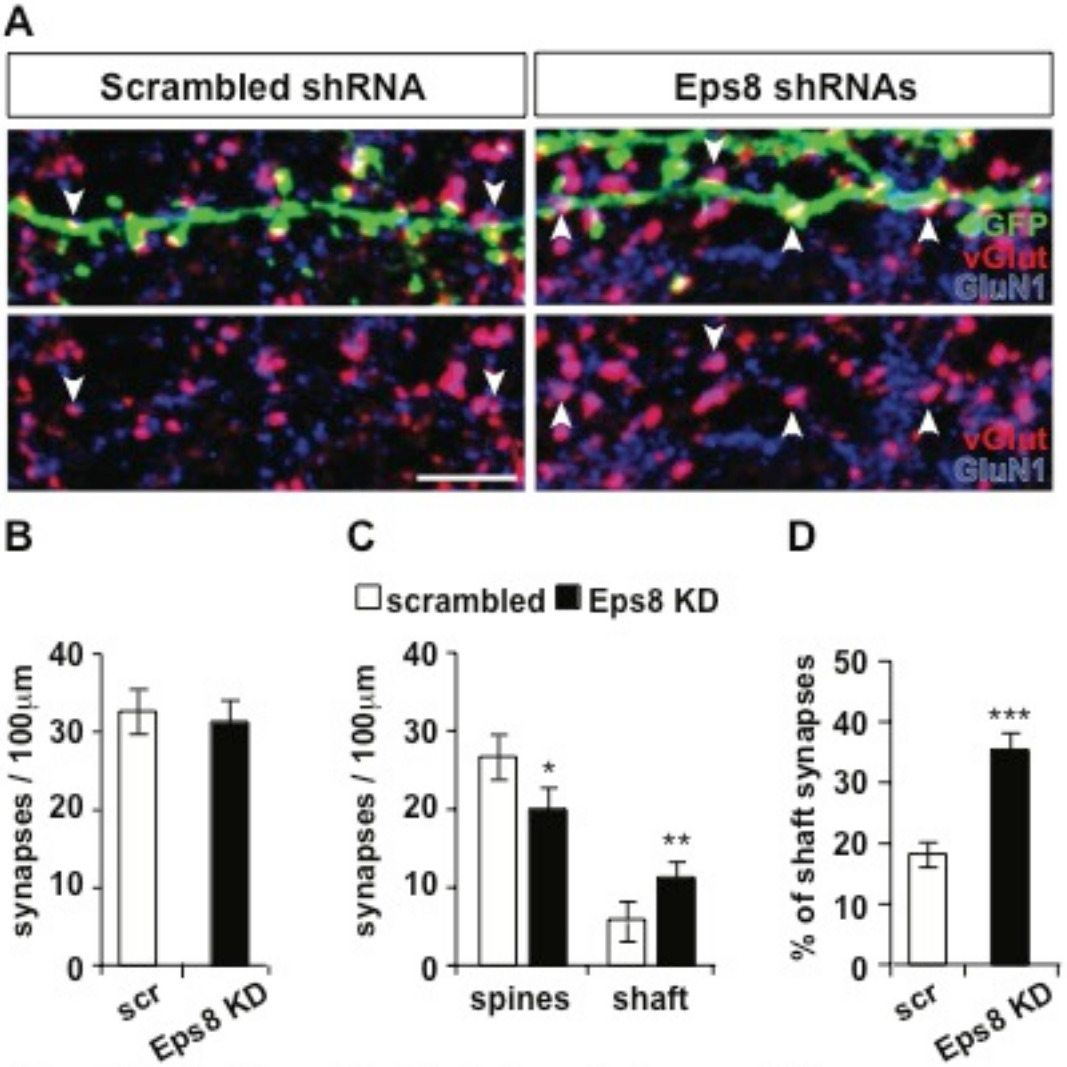



**Figure 6.4: Eps8 regulates the balance between excitatory synapses on spines and on the dendritic shaft.** Eps8 KD does not affect the total number of synapses, but it does decrease the number of synapses on spines (A), whereas increases the number of shaft synapses (A - arrowheads) containing GluN1 (B, C and D). Scale bar: 5µm. *p<0.05, **p<0.01, ***p<0.001.

To investigate the functional consequences of Eps8 loss of function at synapses, we performed whole-cell patch-clamp recordings and measured AMPAR-mediated miniature excitatory postsynaptic currents (mEPSCs) in neurons expressing control scrambled and Eps8 shRNAs (Figure 6.5E). We found that Eps8 KD did not affect the frequency or the amplitude of mEPSCs (Figure 6.5F and 6.5G, scr: 18; Eps KD: 20 cells). These results are consistent with our findings that Eps8 is required for the localisation of excitatory synapses on dendritic

spines but it does not affect the total number GluA1-labelled synapses, therefore not affecting basal synaptic transmission on the neuron.

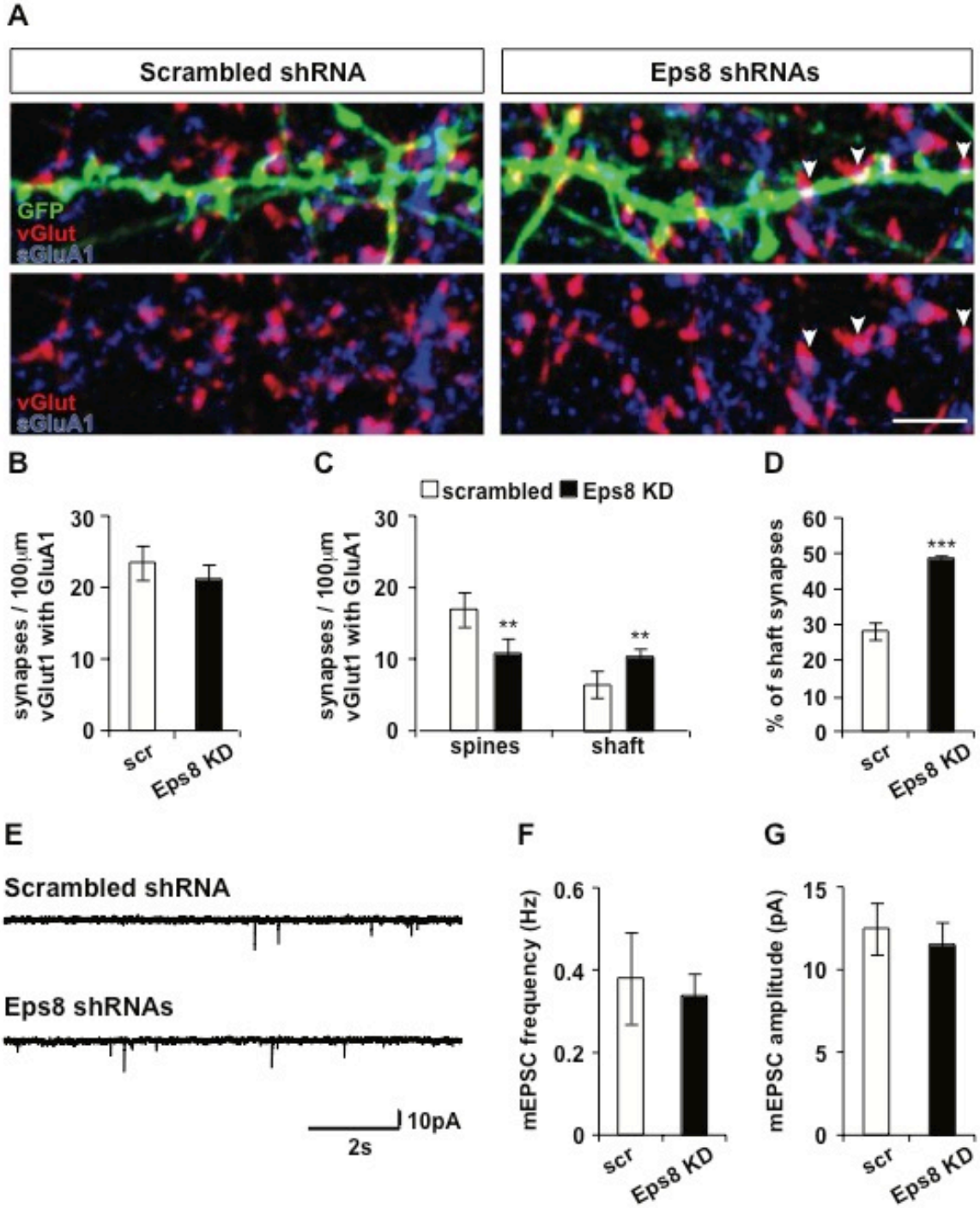



**Figure 6.5: Loss of function of Eps8 does not affect the density of AMPA receptor at excitatory synapses or basal synaptic transmission.** (A-D) Eps8 KD does not affect the total number of synapses containing surface GluA1, but it induces a decrease in the number of synapses on spines with a concomitant increase in the number of shaft synapses (A - arrowheads) containing surface GluA1. Scale bar: 5μm. (E) AMPA receptor-mediated mEPSCs were recorded from cultured neurons. Eps8 knockdown does not affect the frequency (F) or the amplitude (G) of mEPSCs. Representative 10s traces of mEPSCs illustrating similar frequency and amplitude in scrambled and Eps8 shRNA-expressing cells are depicted. **p<0.01, ***p<0.001.

### 6.2.4 Eps8 is required for LTP-dependent excitatory synapse formation.

Neuronal activity plays a crucial role in the formation and modulation of neuronal circuits. Several studies have demonstrated that long-term potentiation (LTP) increases both the number and size of dendritic spines (Alvarez & Sabatini 2007, Bosch & Hayashi 2011, Segal 2005). Actin cytoskeleton dynamics are required for activity-mediated spine morphological changes (Bramham 2008). We therefore decided to examine the contribution of Eps8 to LTP-mediated spine plasticity. To address this question, we used a chemical LTP (cLTP) protocol, which consists of the activation of NMDA receptors with glycine in the absence of $Mg^{2+}$. As previously reported (Fortin et al 2010, Keith et al 2012), this protocol induces spine formation and enlargement. Indeed, in control scrambled shRNA-expressing neurons, cLTP significantly increases both the number and the size of dendritic spines (Figure 6.6A, 6.6B and 6.6C, scr cnt: 24; scr cLTP: 21 cells). In contrast, Eps8 shRNAs-expressing neurons do not respond to the cLTP stimulus, as spine density and size remain unchanged (Figure 6.6A, 6.6B and 6.6C, Esp8 KD cnt: 25; Eps8 cLTP: 26 cells). Thus, Eps8 is required for LTP-dependent structural plasticity of dendritic spines.

To examine the role of Eps8 on LTP-mediated functional plasticity, we recorded AMPAR-mediated mEPSCs (Figure 6.7A). We found that cLTP increases both mEPSC frequency (180%) and amplitude (48%) in scrambled shRNA-expressing cells (Figure 6.7B and 6.7C, scr cnt: 21; scr cLTP: 14 cells), as previously shown in cultured hippocampal neurons (Fortin et al 2010, Keith et al 2012, Oh & Derkach 2005). In contrast, Eps8 silencing completely blocks the effect of cLTP on mEPSC frequency. However, the amplitude was significantly increased by 38% after cLTP (Figure 6.7B and 6.7C, Esp8 KD cnt: 17; Eps8 cLTP: 21 cells), as observed in scrambled shRNA-expressing neurons. These results indicate that Eps8 is required for LTP-mediated synapse formation, but not for LTP-induced synaptic strengthening.

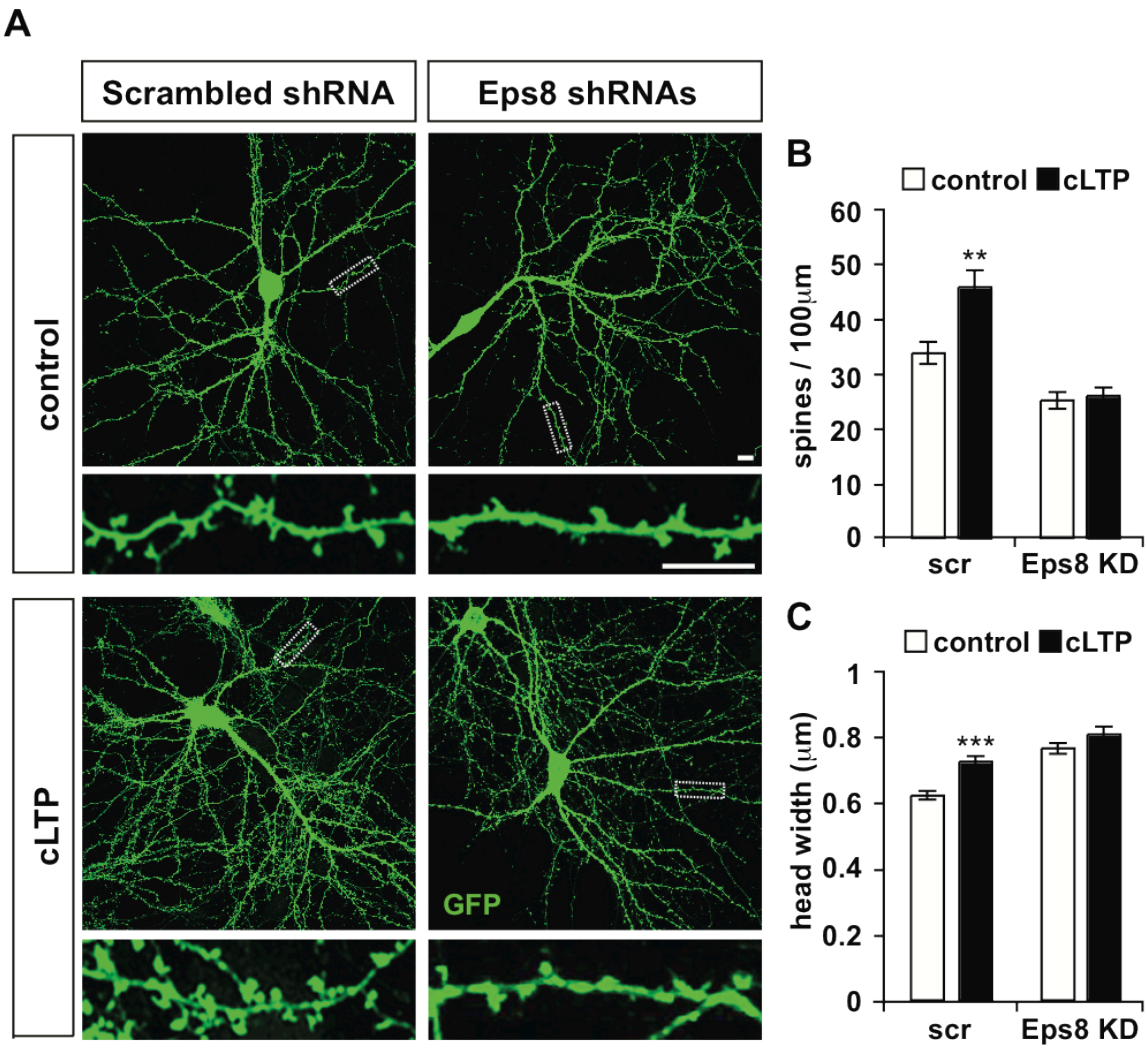



**Figure 6.6: Eps8 is required for activity-dependent structural plasticity.** cLTP was induced in hippocampal neurons expressing scrambled shRNA or shRNAs against Eps8 (A). Scale bars: 10μm. In scrambled shRNA-expressing cells cLTP increases spine density (B) and size (C). In neurons expressing Eps8 shRNAs, cLTP does not affect the number or the size of dendritic spines (B and C). *p<0.05, **p<0.01, ***p<0.001, ns: non-significant.

## 6.3 DISCUSSION

Long-term changes in synaptic activity have profound effects on the formation and morphology of dendritic spines and synaptic strength (Alvarez & Sabatini 2007, Bosch & Hayashi 2011, Kasai et al 2010, von Bohlen Und Halbach 2009). Indeed, LTP increases spine density and spine growth through modifications of the actin cytoskeleton (Bosch & Hayashi 2011, Cingolani & Goda 2008, Hotulainen & Hoogenraad 2010). However, little is known about the molecular mechanisms by which neuronal activity regulates the actin cytoskeleton during spine plasticity. Here, we demonstrate that the actin-capping protein Eps8 modulates spine morphogenesis and is required for LTP-mediated spine formation and some aspects of synaptic potentiation.

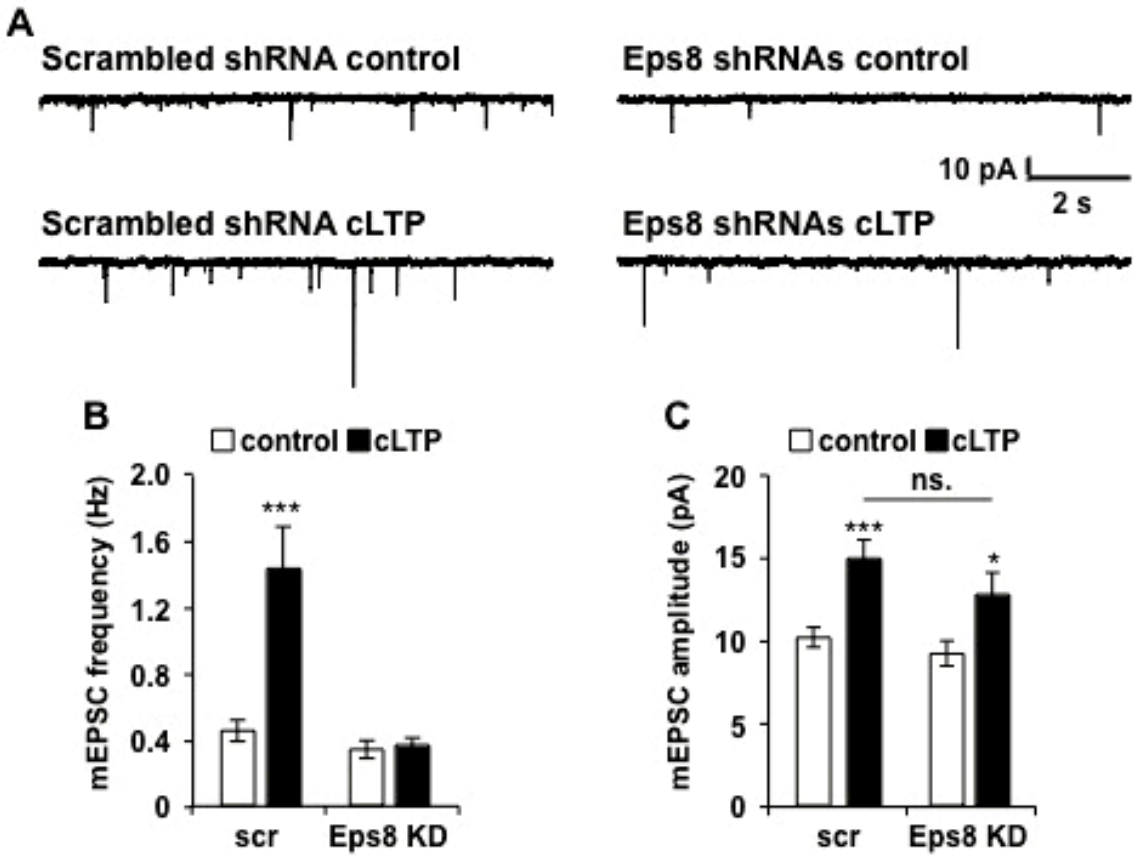



**Figure 6.7: Eps8 is required for activity-dependent functional plasticity.** cLTP was induced in hippocampal neurons expressing scrambled shRNA or shRNAs against Eps8 (A). Scale bars: 10μm. In scrambled shRNA-expressing cells cLTP increases spine density (B) and size (C). In neurons expressing Eps8 shRNAs, cLTP does not affect the number or the size of dendritic spines (B and C). (D) AMPA receptor-mediated mEPSCs were recorded from cultured neurons in control condition or after cLTP induction. Representative 10s traces of mEPSCs are depicted (E and F). Scrambled shRNA-expressing cells show a significant increase in both mEPSC frequency and amplitude. In contrast cLTP in Eps8 KD has no effect in mEPSC frequency (E), but induces a significant increase in the amplitude of mEPSCs (F).. *p<0.05, **p<0.01, ***p<0.001, ns: non-significant.

Eps8 is a multifunctional protein that induces actin remodelling through its bundling and capping activities or through Rac1 activation (Disanza et al 2004, Disanza et al 2006, Hertzog et al 2010, Menna et al 2009, Roffers-Agarwal et al 2005). Interestingly, Eps8 can either induce or inhibit filopodium formation (Disanza et al 2006, Menna et al 2009). These activities are highly dependent on the cellular context and are regulated by interactions with its partners IRSp53 and Abi1/2 (Vaggi et al 2011). In HeLa cells, Eps8 promotes filopodium formation through the interaction with IRSp53 via its actin bundling activity (Disanza et al 2006, Vaggi et al 2011). In neurons in contrast, Eps8 inhibits filopodium formation through its capping activity (Menna et al 2009). Consistent with this finding, we show that Eps8 inhibits filopodium formation in dendrites. These

results suggest that Eps8 does affect actin bundling activity during the formation of neuronal filopodia.

In neurons, Eps8 is not required for Rac1 activation, as Rac activity is not affected in Eps8 KO mice (Menna et al 2009). In addition, Rac1 inhibition did not mimic the effect of Eps8 loss of function in the formation of axonal filopodia in neurons (Kozma et al 1997, Menna et al 2009). Consistent with these findings, we found that the Rac1 inhibitor (NSC23766) did not block Eps8 function on spines (data not shown). In addition, we found that a mutant Eps8 (Eps8TM), defective in actin capping but not in Rac1 activation, mimics the loss of function of Eps8. Together, these results suggest that Eps8 regulates spine morphogenesis through a pathway independent of Rac1.

Barbed end actin-capping proteins are of major importance in the regulation of actin dynamics by inhibiting actin filament elongation. Defects in capping proteins result in the formation of long actin filaments and promote excessive filopodium formation (Fan et al 2011, Mejillano et al 2004, Menna et al 2009). Indeed, Eps8 loss of function increases the amount of filamentous actin (Vaggi et al 2011) and axonal filopodia in neurons (Menna et al 2009). In agreement with these findings, we show that Eps8 loss of function increases, whereas gain of function decreases, the number of dendritic filopodia in hippocampal neurons. Our loss of function studies demonstrate that Eps8 regulates the number of dendritic spines. Moreover, Eps8 KD results in bigger spines with irregular shape and filopodial protrusions emerging from spine heads. The structural effects of Eps8 KD on dendritic filopodia and spines are similar to those observed when CP, an actin-capping protein, is silenced (Fan et al 2011). These findings are consistent with the view that Eps8 acts as a capping protein to regulate filopodium and spine formation.

Several experiments demonstrate that Eps8 regulates spine morphogenesis through its actin capping activity. First, a mutant Eps8 carrying three point mutations (V729A, T731A and W732A), that specifically abolish its capping activity (Menna et al 2009), exhibits defects in spine morphogenesis mimicking the loss of function of Eps8. Second, Eps8 loss of function increases F-actin accumulation at dendritic spines as expected for a capping protein. Third, free-barbed end assays and FRAP experiments demonstrate that Eps8 KD increases

the level of uncapped barbed-ends available for actin polymerization and induces fast actin turnover within dendritic spines. Together our results strongly suggest that Eps8 regulates spine formation and morphology through its actin capping activity.

Although most excitatory synapses are formed on dendritic spines, some synapses are present on the dendritic shaft. However, very little is known about the mechanisms that regulate the formation of spine versus shaft synapses. For example, silencing of neurobeachin, a protein that regulates membrane trafficking, decreases spine density with a concomitant increase in the number of excitatory shaft synapses (Niesmann et al 2011). Similar effects are observed in the gain of function of Rap2, a small GTPase (Fu et al 2007). Here, we showed that Eps8 KD induces the localization of excitatory synapses on the dendritic shaft with a concomitant decrease in the number of synapses on spines. Several studies have shown that increased shaft synapse density does not affect spontaneous synaptic currents, when the total number of synapses is unchanged (Aoto et al 2007, Fu et al 2007, Ivenshitz & Segal 2010, Niesmann et al 2011). Indeed, we found that Eps8 KD does not affect the frequency or the amplitude of mEPSCs in hippocampal neurons under basal conditions. Consistent with our findings, spontaneous synaptic currents are unaffected in cerebellar granule neurons (CGNs) from Eps8 KO mice (Offenhauser et al 2006). In summary, our findings demonstrate that Eps8 regulates the balance between spine and shaft excitatory synapses without affecting basal synaptic transmission.

Activity-dependent changes in spine plasticity are highly dependent on local changes in the actin cytoskeleton (Fukazawa et al 2003, Gu et al 2010, Honkura et al 2008, Okamoto et al 2004). Indeed, neuronal activity regulates actin turnover within dendritic spines (Honkura et al 2008, Okamoto et al 2004, Star et al 2002). Barbed-end capping proteins regulate actin turnover by inhibiting filament elongation, therefore maintaining the equilibrium between monomeric (G-actin) and filamentous actin (F-actin). Although, the role of capping proteins in spine morphogenesis has been documented (Fan et al 2011, Gao et al 2011), their function in activity-mediated changes has not been reported. Here, we demonstrate that Eps8 silencing suppresses the effect of cLTP on spine morphogenesis demonstrating that Eps8 is required for activity-mediated spine structural plasticity.

Eps8 is required for some aspects of LTP-mediated synaptic plasticity. We found that Eps8 KD impairs LTP-mediated increase in the frequency but not in the amplitude of AMPAR-mediated mEPSCs suggesting that in the absence Eps8, an LTP stimulus can still increase the density and/or function of AMPARs at the synapse. Interestingly, previous studies have shown that actin polymerisation is crucial for the maintenance, but not the induction of LTP (Fukazawa et al 2003, Krucker et al 2000, Ramachandran & Frey 2009). Moreover, it has been suggested that different pools of actin are present at spines to differentially regulate different processes (Honkura et al 2008, Okamoto et al 2004, Star et al 2002). Based on these findings, we propose that Eps8, through its capping activity, regulates a pool of actin required for the late stages of LTP without affecting LTP-mediated increases in synaptic strength.

In summary, we demonstrated that Eps8 is required for spine morphogenesis. In addition, we show that Eps8 regulates actin polymerization and turnover within dendritic spines. Interestingly, Eps8 KD induces a shift towards excitatory synapses on the dendritic shaft, an effect contrary to this observed in Eps8 gain of function (Chapter 5). Importantly, Eps8 is required for structural and functional plasticity of synapses induced by long-term potentiation.

# Chapter 7:

# General Discussion

Wnt proteins are well known secreted factors that promote the formation of neuronal circuits (Budnik & Salinas 2011, Park & Shen 2012, Rosso & Inestrosa 2013). During the last decade, significant progress has been made in understanding the function/role of Wnts in synapse formation and function. However, we have much less understanding on the molecular mechanisms involved in these processes. Previous studies from our lab have demonstrated Wnt3/3a induces axonal remodelling of NT-3 responsive dorsal root ganglia neurons through modifications in the microtubule and actin cytoskeleton (Krylova et al 2002, Purro et al 2008);Hoyos-Flight, PhD thesis 2005). In addition, Wnt7a promotes the growth of dendritic spines, actin-rich protrusions that receive excitatory input, through postsynaptic activation the Dvl1-CaMKII signalling (Ciani et al 2011). However, the pathways that are downstream of Wnt3/3a and Wnt7a to regulate the actin cytoskeleton remain unknown.

The main aim of this thesis was to identify the molecular pathway(s) that are activated Wnt signalling to promote axonal remodelling and spine growth. To achieve this, I performed a yeast two-hybrid screen using Dvl1 – a scaffold protein required for Wnt-mediated responses - as a bait. I found that the actin-binding protein Eps8 interacts directly with Dvl1. I then aimed to examine whether Eps8 has a role in axonal remodelling and spine morphogenesis. Our results revealed that Eps8 induces growth cone enlargement independently of its actin binding domain. Importantly, Eps8 is required for Wnt3a-mediated axonal remodelling. On the postsynaptic side, Eps8 promotes spine morphogenesis through its actin-capping activity and is required for structural and functional plasticity of dendritic spine through a pathway that is probably independent of Wnt.

## 7.1 Eps8 role in axonal remodelling

Previous studies have demonstrated that Wnts signal through a divergent canonical Wnt pathway to induce changes in the microtubule cytoskeleton during growth cone remodelling (Miech et al 2008, Purro et al 2008). However, detailed examination of the actin cytoskeleton revealed that Wnts also increase actin dynamics during axonal remodelling (Hoyos-Flight, PhD thesis 2005). Here we examined the molecular mechanisms involved in Wnt3a-mediated axonal remodelling. We found that Wnt3a signalling through Dvl1 and inhibition of

Gsk3β regulates the actin cytoskeleton in growth cones (Chapter 4). My data suggest that activation of a Wnt divergent canonical pathway is responsible for both actin and microtubule cytoskeletal changes during axon terminal remodelling.

Our previous studies demonstrated that Wnt3a promotes F-actin accumulation during axonal remodelling by inducing the formation of highly dynamic actin filaments in growth cones (Hoyos-Flight, PhD thesis 2005). These findings support the notion that Wnt3a could induce actin polymerization by promoting the loss of an actin-capping protein – such as Eps8 - from the filament barded-ends, an effect similar to the one seen with APC removal from microtubule plus-ends (Purro et al 2008). However, we found that gain of function of Eps8 mimics the effect of Wnt3a on growth cone enlargement in growth cones, whereas Eps8 loss of function impairs Wnt3a-mediated axonal remodelling. In addition, we showed that Eps8 induces F-actin accumulation in growth cones, an effect not consistent for an actin-capping protein. Finally, a mutant Eps8 that lacks the actin-binding domain, therefore cannot bind directly to actin filaments, induces growth cone enlargement and accumulation of F-actin similar to full-length Eps8 (Chapter 4). Altogether, these results demonstrate that Eps8 is required for Wnt3a to induce axonal remodelling, but not through its direct binding to actin.

How does Eps8 contribute to Wnt-mediated remodelling? Eps8 enhances cell proliferation and migration through the PI3K-Akt pathway and increases β-catenin levels (Sala & Segal 2014, Wang et al 2009, Wang et al 2010). These results suggest that Eps8 could promote axonal remodelling by activating the canonical Wnt pathway through Akt signalling. Importantly, expression of a constitutively active form of Akt in DRG neurons increases branching and growth cone size (Grider et al 2009), mimicking the effects observed upon activation of the Wnt3a-Dvl1-Gsk3β pathway (Purro et al 2008). Given that Eps8 interacts with Dvl1, which is upstream of Gsk3β, our findings raise the possibility that Wnt3a regulates axonal remodelling through a pathway where interaction of Eps8 with Dvl1 activates Akt, leading to Gsk3β inhibition and resulting in growth cone enlargement and axon remodelling (Figure 7.1). However, the role of Wnt signalling in Akt activation has not been well characterised. Currently, very few papers have reported that Akt is downstream of Wnt signalling (Constantinou et

al 2008, Fukumoto et al 2001, von Maltzahn et al 2012a). In PC12 cells, expression of Wnt1 or Dvl1 increased Akt activity (Fukumoto et al 2001). Similarly, exposure to Wnt3a or Wnt7a resulted in Akt activation in fibroblast-like cells and differentiated myofibres, respectively (Constantinou et al 2008, von Maltzahn et al 2012a). Further studies are required to understand whether Wnts activate Akt signalling in neurons and in particular during axonal remodelling.

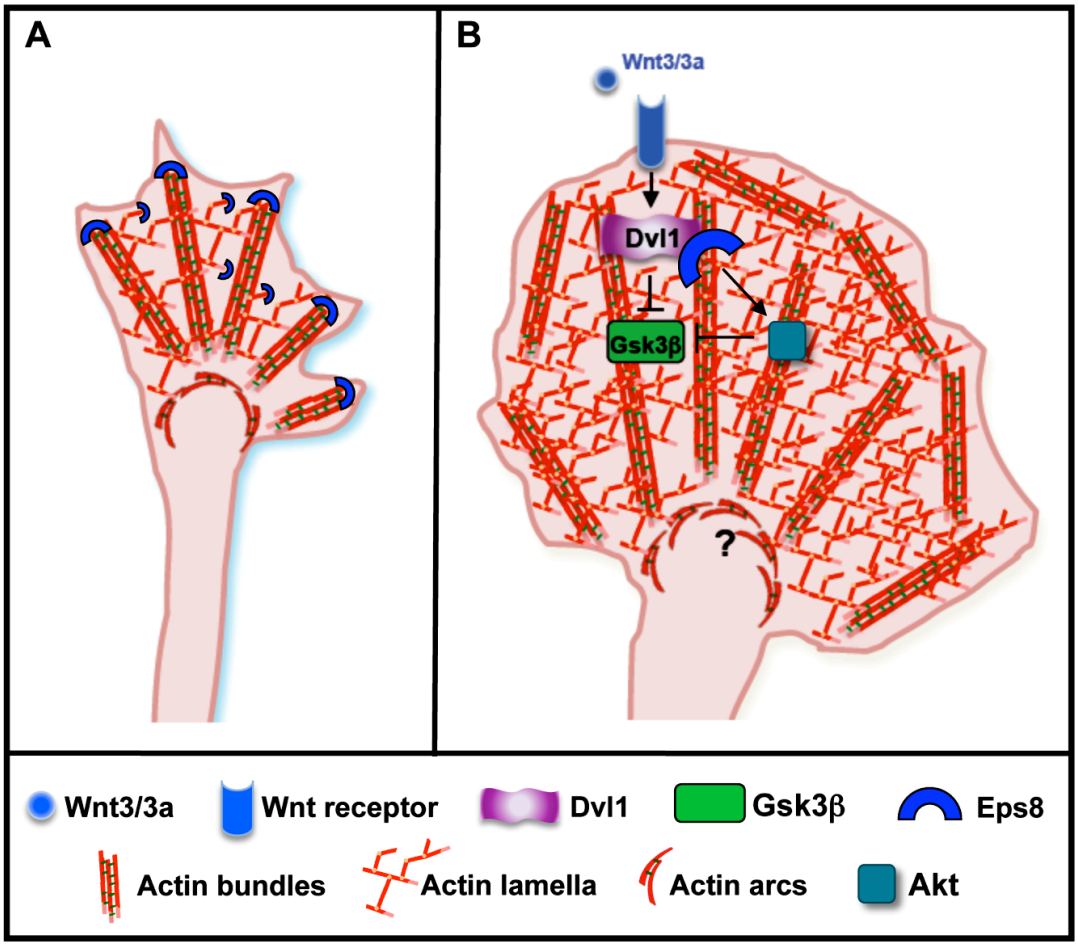



**Figure 7.1: Proposed model for the role of Eps8 in Wnt3a-mediated axonal remodelling.** (A) In the absence of Wnt factors Eps8 binds and caps the barbed-ends of actin filaments and inhibits their elongation. (B) Wnt3/3a induces a densely packed and disorganized actin network with several actin filaments aligned parallel to the leading edge. These effects are possibly mediated though Gsk3β inhibition induced by Eps8-Akt signalling. The effect of Wnt3a on actin arcs has to be examined in detail, as their role is to inhibit microtubule entry in the growth cone peripheral region.

In summary, previous results from our lab have shown that Wnt3a induces actin cytoskeletal re-arrangements during the remodelling of DRG growth cones. Here, we show that the Dvl1-interacting protein Eps8 mimics the effect of Wnt3a in F-actin accumulation and axonal remodelling. Importantly, Eps8 is required for

Wnt3a-mediated growth cone enlargement and axonal remodelling. However, the effects of Eps8 in growth cones are independent of its actin-binding region, indicating that Eps8 regulates the actin cytoskeleton during axonal remodelling through an indirect mechanism.

## 7.2 Eps8 role in spine morphogenesis

Eps8 can either promote or inhibit filopodium formation, due to its capping and bundling activities (Disanza et al 2006, Menna et al 2009). Interaction with IRSp53 promotes side filament binding and leads to the formation of filopodia in HeLa cells via its bundling activity (Disanza et al 2006, Vaggi et al 2011). On contrary in neurons, Eps8 interacts mainly with Abi1 and inhibits the formation of axonal filopodia through actin capping (Menna et al 2009, Vaggi et al 2011). Based on gain and loss of functions studies we demonstrated that Eps8 inhibits the formation of dendritic filopodia (Figure 7.2). Together these findings exclude the possibility that Eps8 induces the formation of dendritic filopodia through actin bundling.

In contrast to filopodium formation, Eps8 promotes the formation of dendritic spines (Figure 7.2). Our studies revealed that Eps8 increases spine density by promoting the formation of stubby spines. On the other hand, Eps8 silencing leads to fewer small spines with a concomitant increase in the number of large spines, resulting in an average increase in spine head size. In addition, loss of function of Eps8 leads to irregular spine shape with spine head filopodial protrusions (Figure 7.2). These findings demonstrate that Eps8 is required for spine morphogenesis and suggest that Eps8 regulates *de novo* spine formation, since loss of function of Eps8 results to the loss of small spines, which are most probably those that represent the newly formed.

The structural effects of Eps8 KD on dendritic filopodia and spines are similar to those observed when CP, an actin-capping protein, is silenced (Fan et al 2011). These findings suggest that Eps8 acts as a capping protein to regulate filopodium and spine formation. Indeed, we found that a mutant Eps8 carrying three point mutations (V729A, T731A and W732A), that specifically abolish its capping activity (Menna et al 2009), exhibits defects in spine morphogenesis mimicking the loss of function of Eps8. Also, KD increases F-actin accumulation at dendritic

spines as expected for a capping protein. Finally, my free-barbed end assays and FRAP experiments demonstrate that Eps8 KD increases the level of uncapped barbed-ends available for actin polymerization and induces fast actin turnover within dendritic spines (Figure 7.3). Together, our results demonstrate that Eps8 regulates spine formation and morphology through its actin capping activity.

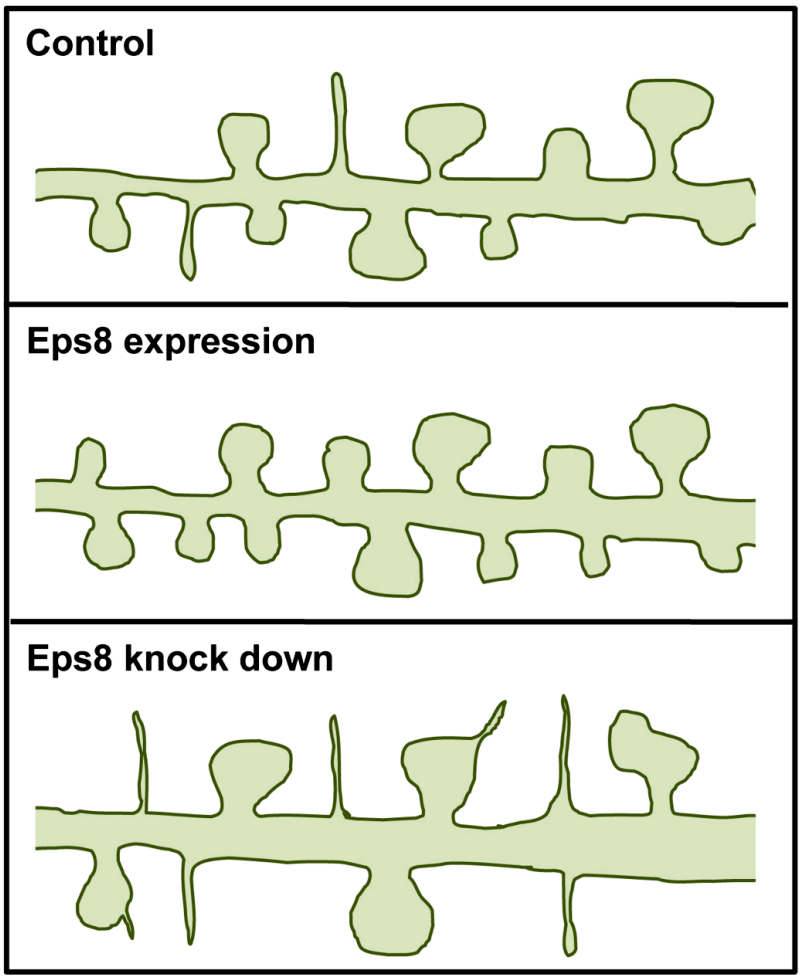



**Figure 7.2: Eps8 promotes spine morphogenesis.** Eps8 gain of function promotes the formation of stubby spines with a concomitant decrease in the number of filopodia. In contrast, Eps8 knock down results in increased filopodium density and fewer spines with irregular shape.

## 7.3 Role of Eps8 in synaptogenesis

During synaptogenesis, most synapses are initially formed on the dendritic shaft resulting in the subsequent formation of spine synapses. The localisation of synapses on dendritic spines offers strict compartmentalisation of synaptic signalling, but also they offer protection from excitotoxicity due to $Ca^{2+}$ influx upon the arrival of an action potential (Segal 2010). However, very little is

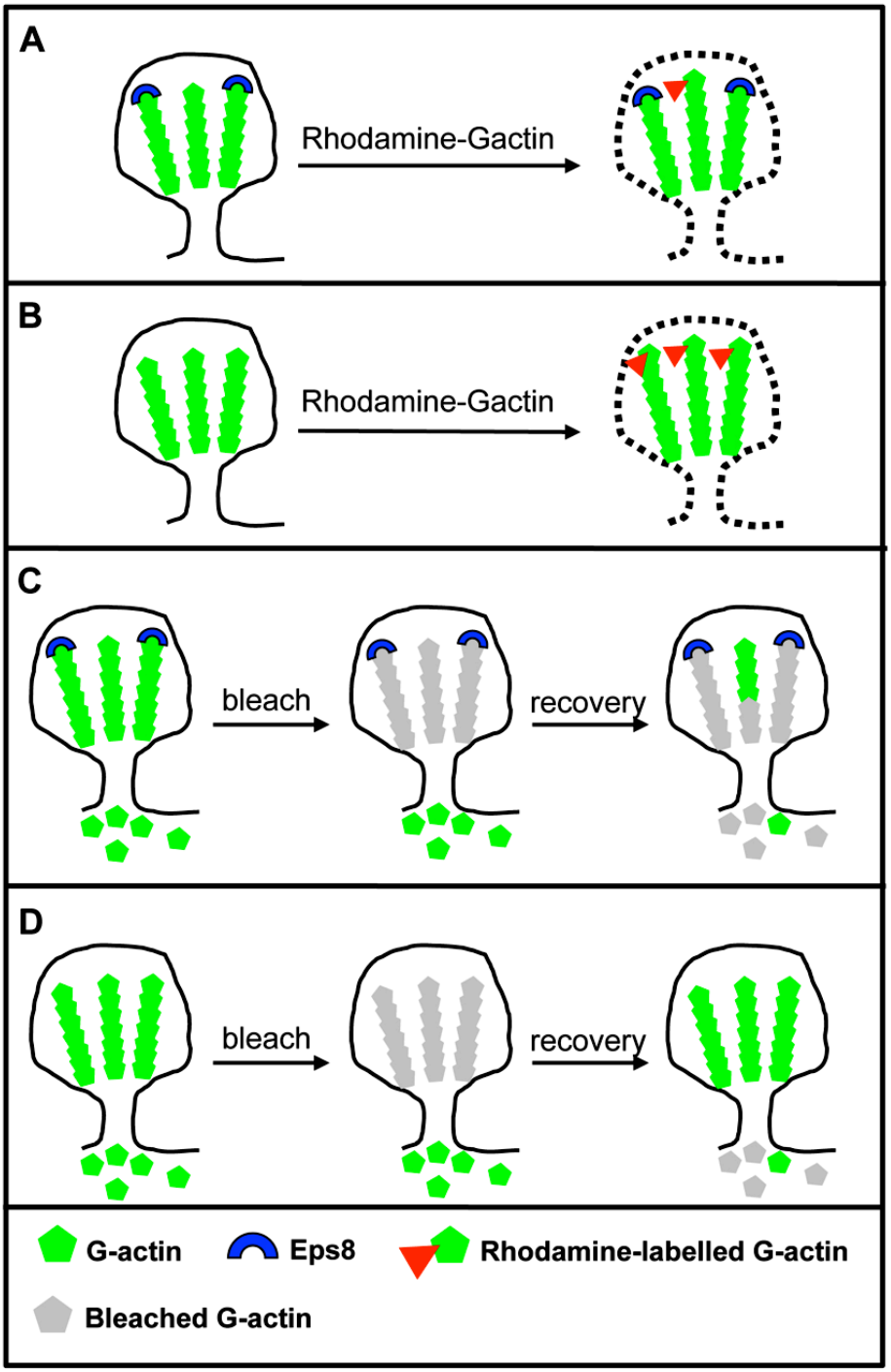



**Figure 7.3: Eps8 is acting as a capping protein in dendritic spines.** Free barbed-end assays (A and B) revealed that loss of Eps8 (B) leads to increased actin polymerization due to higher rate of G-actin incorporation in the barbed ends of actin filaments. FRAP experiments (C and D) demonstrated that Eps8 silencing increases actin turnover within dendritic spines.

known about the mechanisms that regulate the formation of spine versus shaft synapses. Our gain and loss of function experiments demonstrate that Eps8 induces a shift of excitatory synapses from the dendritic shaft to spines, without affecting synapse density (Figure 7.4). Consistently, Eps8 KD did not affect the frequency of AMPAR-mediated EPSCs confirming that synapse density remains

unchanged. These results suggest that Eps8 by promoting the localisation of glutamatergic synapses to dendritic spines could increase neuroprotection from high $Ca^{2+}$ levels. Interestingly, loss of function of Gelsolin, another capping protein, increases glutamate-induced neurotoxicity (Furukawa et al 1997). Together these findings support the possibility that actin-capping proteins are protecting the neuron by regulating excitatory synapse localisation on dendritic spines.

Spine size has been correlated with their content in AMPARs (Matsuzaki et al 2001). Although silencing of Eps8 results in spine enlargement, we did not find any changes in the volume or number of surface GluA1 clusters per single spine (data not shown). These results are consistent with our electrophysiology recordings showing that the amplitude of AMPAR-mediated mEPSCs is unaffected in Eps8shRNA-expessing cells. Thus, ours findings demonstrate that spine size and AMPAR content are not directly coupled and that under basal conditions changes in actin dynamics by loss of function of Eps8 does not affect AMPAR delivery to synapses. In addition, these results shows that the ratio of AMPAR content to spine volume (number of AMPARs/spine volume) is decreased upon loss of function of Eps8, which could result in lower concentration of $Na^{+}$ and $K^{+}$ - and $Ca^{2+}$ in the case of GluA2 lacking receptors - upon AMPAR activation. Thus, neurons lacking Eps8 will sense to a smaller extent glutamate released frominputs located on dendritic spines. On the other hand, loss of Eps8 leads to an increase in shaft synapse formation. Previous studies have shown that excitatory synapses located on the dendritic shaft are likely to produce larger synaptic currents than spine synapses (Segal 2010). This finding supports the notion that neurons lacking Eps8 will respond more to inputs received at the dendritic shaft than those received on the dendritic spines.

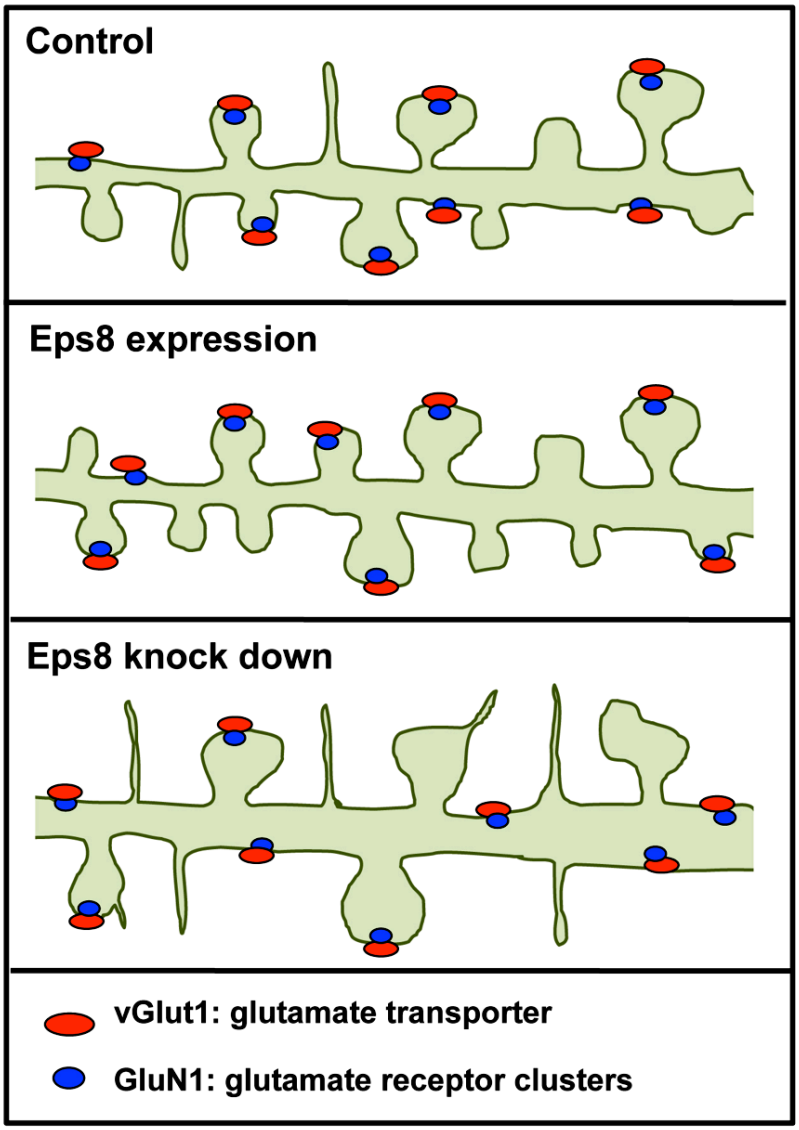



**Figure 7.4: Eps8 regulates the localisation of excitatory synapses on dendritic spines.** Eps8 gain or loss of function does not affect synapse density. However, Eps8 promotes the number of synapses localised on dendritic spines with a concomitant decrease in shaft synapses. In contrast Eps8 knock down results in more shaft synapses with a concomitant decrease in excitatory synapses located on spines.

## 7.4 Eps8 in structural & functional plasticity

Activity-dependent changes in structural and functional plasticity are highly dependent on local changes in the actin cytoskeleton (Fukazawa et al 2003, Gu et al 2010, Honkura et al 2008, Okamoto et al 2004). In particular, neuronal activity regulates actin turnover (Honkura et al 2008, Star et al 2002) and the ratio of F-actin / G-actin within dendritic spines (Okamoto et al 2004). Using loss of function approaches we demonstrated that Eps8 is required for activity-

mediated spine structural plasticity, since in neurons that lack Eps8 spine density and size remain unchanged.

Actin-capping proteins inhibit the elongation of actin filaments through binding at the barbed-ends. Defects in capping proteins result in the formation of long actin filaments and promote excessive filopodium formation (Fan et al 2011, Mejillano et al 2004, Menna et al 2009). Indeed, Eps8 loss of function increases the amount of F-actin within dendritic spines resulting in the formation of filopodial protrusions on spine heads. In addition, Eps8 silencing results in increased number of free barbed-ends within spines (Chapter 6). These results show that the content of F-actin in spines is increased in the absence of Eps8 and suggest that the amount of G-actin is probably decreased. Therefore, the impairment in structural plasticity upon Eps8 silencing could be due to the depletion of the G-actin pool available for polymerization.

In addition to structural plasticity upon LTP, synapses undergo functional changes, which are reflected by an increase in the frequency and amplitude of AMPAR-mediated EPSCs. We thus examined whether Eps8 is required for functional plasticity using a protocol that induces NMDAR-dependent synaptic potentiation (Fortin et al 2010, Lu et al 2001, Molnar 2011, Oh & Derkach 2005). This protocol depends on the unsilencing of existing synapses through the insertion of AMPAR, without any reported changes on the formation of new presynaptic sites. We found that Eps8 KD impairs LTP-mediated increase in the frequency, but not the amplitude of AMPAR-mediated mEPSCs. These results suggest that in the absence Eps8, LTP protocols can still increase the density or the functional properties of AMPARs in a single synapse, therefore leading to an increase in mEPSCs amplitude, but synapses that lack AMPARs cannot become unsilenced (Figure 7.5). Interestingly, it has been suggested that different pools of actin are present at spines to differentially regulate different processes (Honkura et al 2008, Okamoto et al 2004, Star et al 2002). Based on these findings, Eps8 could regulate a pool of actin required for the delivery of AMPARs at silent synapses upon increase in neuronal activity through exocytosis or the lateral movement of AMPARs from extrasynaptic sites.

Several pieces of evidence support a role for the actin-severing factor Cofilin in AMPAR trafficking. A recent study showed that elevated Cofilin activity

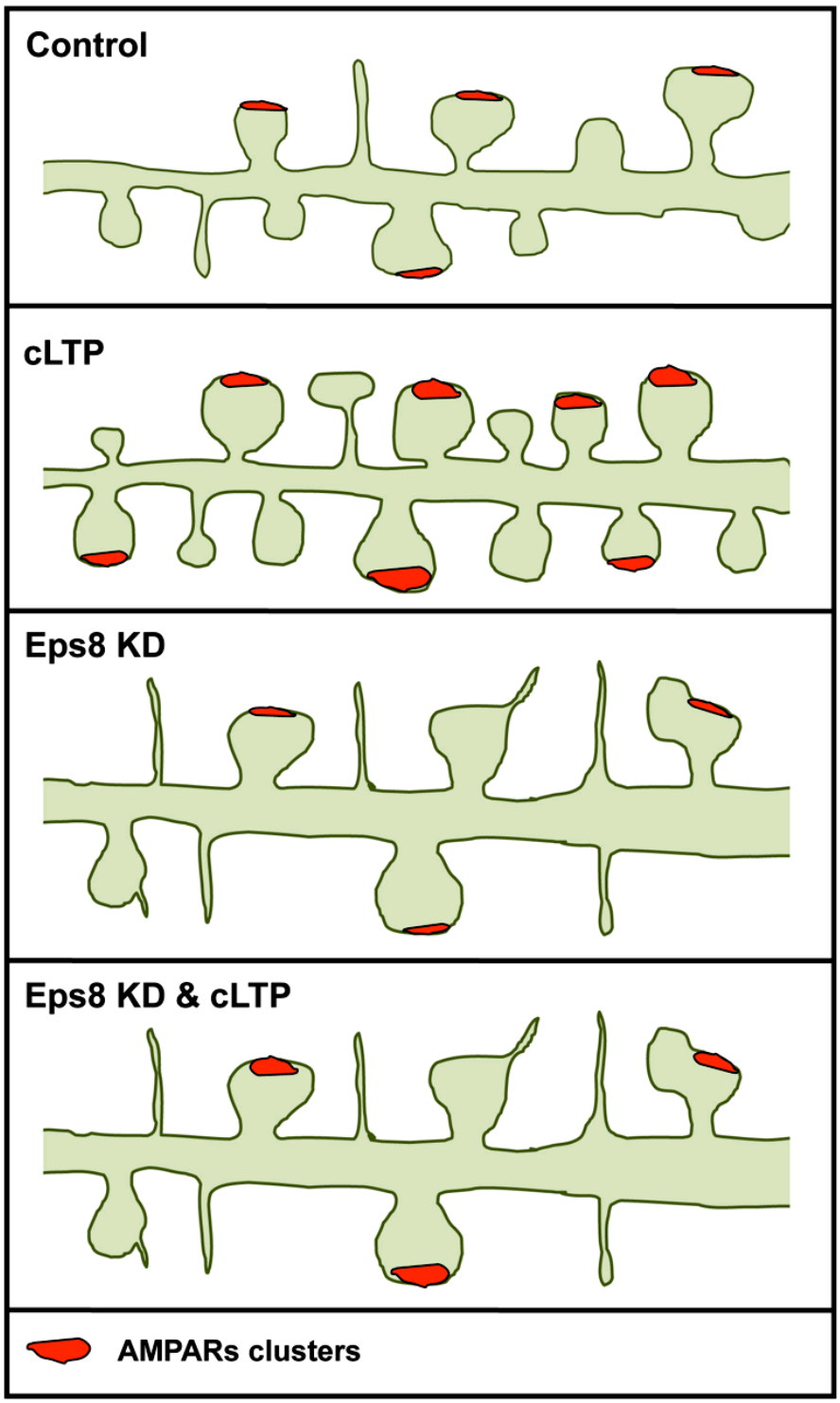



**Figure 7.5: Eps8 is required for activity dependent structural plasticity and synapse formation.** Eps8 loss of function impairs cLTP-induced structural plasticity. In addition, recordings of AMPAR-mediated mEPSCs revealed that activity-dependent synapse formation is defected. On contrary, cLTP-mediated synapse strengthening is normal, suggesting that existing synapses can be potentiated in the absence of Eps8, whereas silent synapses cannot became unsilenced.

(dephosphorylation), induced during memory extinction, promotes GluA1 and GluA2 translocation to synapse and their insertion into the postsynaptic membrane (Wang et al 2013). Another study found that during cLTP Cofilin

undergoes transient activation and promotes GluA1 membrane insertion in dendritic spines (Gu et al 2010). Importantly, studies in Cofilin knockout neurons revealed that GluA2 lateral diffusion to the synaptic sites is defected (Rust et al 2010). Altogether these findings demonstrate that increased Cofilin activity promotes, whereas inhibition of Cofilin activity impairs AMPAR delivery to the synapse.

Interestingly, Eps8 is required for activation of Cofilin upon glutamate treatment in cerebellar granule neurons (CGNs), but not under basal conditions. NMDARs induce a negative feedback loop due to an increase in intracellular $Ca^{2+}$, which leads to the dephosphorylation and activation of Cofilin, resulting in actin disassembly (Sarmiere & Bamburg 2004, Wang et al 2005). This pathway is disrupted in neurons lacking Eps8 (Offenhauser et al 2006). Based on these findings authors proposed a model where Eps8 lies downstream of NMDARs and upstream of Cofilin activation (Offenhauser et al 2006). This model could explain why Eps8 silencing completely blocks the effect of cLTP on mEPSC frequency, as loss of function of Eps8 would impair activation of Cofilin, leading to defected AMPAR delivery in synapses. However, more experiments are required to support this hypothesis.

## 7.5 The role of Eps8 in spine morphogenesis & synaptic plasticity *in vivo*

During the writing of this thesis and after the publication of our paper reporting the role of Eps8 in spine formation and plasticity, Mena and colleagues published a study reporting the role of Eps8 in spine and synapse formation *in vivo*. Consistent with our results authors found that Eps8 is required for spine morphogenesis and plasticity through its actin capping activity (Menna et al 2013). However, there are some differences and some novel findings between the two studies that are worthy to be discussed.

### 7.5.1 Eps8 null mice have more immature spines & increased synapse density

Eps8 role in spine morphogenesis was studied using Eps8 null mice both *in vitro*, by preparing hippocampal cultures, and *in vivo* using Golgi staining. The *in vitro* studies show that loss of function of Eps8 decreases the number of mushroom

and stubby type spines with a concomitant increase in the number of thin spines, leading to an overall increase in spine density (Menna et al 2013). However, the images provided show that the "thin spines" are actually filopodia, as they are long dendritic protrusions without a distinguishable head, indicating that Eps8 is required for spine formation. Indeed, Eps8 expression led to an increase in spine density (Menna et al 2013). Together these results show that Eps8 promotes spine formation and inhibits filopodium formation, consistently with our studies. In contrast to the *in vitro* data, Golgi staining revealed that Eps8 null mice possess more and longer spines (Menna et al 2013). However, the images provided are too overexposed to judge whether these dendritic protrusions are spines with immature morphology or filopodia.

The role of Eps8 in synapse formation was also studied by Menna and colleagues. Authors found that Eps8 KO mice have a higher "synaptic area" for Vamp2 and PSD95 staining and conclude that Eps8 null mice have a higher number of synaptic contacts (Menna et al 2013). However, this is the conclusion as the quantification was performed separately for VAMP2 and PSD95, without considering their co-localization, which defines the presence of a synapse. Synapse analyses were also performed *in vitro* using hippocampal cultures from Eps8 null mice. Consistently, with the *in vivo* data authors found that neurons lacking Eps8 have more synapses, as revealed by the apposition of the presynaptic marker vGlut1 and the postsynaptic scaffold protein PSD95 (Menna et al 2013). However, electrophysiological recordings revealed that the frequency of AMPAR-mediated mEPSCs is not affected (Menna et al 2013). These results suggests that at the functional level (presence and function of surface AMPARs) Eps8 is not required for the formation our mature synaptic contacts, consistently with our findings.

In summary, our studies have demonstrated that Eps8 is required for spine formation, but not for synapse formation, whereas Menna et al reported that loss of Eps8 leads to an increase in the formation of synapses and immature spines (Menna et al 2013). These differences could be explained by the experimental approaches that used in these two studies but also due to different interpretation of the data. Our experimental conditions allowed us to examine the role of Eps8 only in the postsynaptic compartment (Figure 7.6). However, Eps8 is also present presynaptically, thus the effects in synapse and spine

formation seen by Menna et al. could be due to the combination of presynaptic and postsynaptic roles of Eps8. Also, the animals used by Menna and colleagues were not conditional knockouts. In contrast, our approach was to the Eps8 silencing experiments were performed on DIV7-8. This means that the effects observed in Menna et al. study could be due to compensation due to the long-term loss of function of Eps8.

### 7.5.2 Eps8 is required for structural & functional plasticity

We found that Eps8 is required for structural and functional plasticity. Accordingly, Menna et al. showed that Eps8 null mice have a defect in learning-dependent spine formation (Menna et al 2013). Importantly, this defect is also reflected at the behavioural level, as Eps8 null mice are impaired in hippocampal-dependent learning and memory (Menna et al 2013). Based on these findings authors examined whether Eps8 is required for synaptic potentiation and found that neurons derived from Eps8 null mice do not show an increase in the frequency or the amplitude of AMPAR-mediated EPSCs upon cLTP compared to control mice. These results demonstrate that Eps8 is required for both synapse formation and synapse strengthening (Menna et al 2013). In contrast, we found that the amplitude of mEPSCs upon cLTP is undistinguishable between control and Eps8shRNA-expressing cells, demonstrating that Eps8 is not required for activity-mediated synapse strengthening. Authors discussed that the difference between our results and theirs could be due to the different approaches. They suggested that the lack of phenotype in the KD of Eps8 could be due to residual levels of Eps8. Although this possibility could not be excluded, there is an intriguing reduction in both the amplitude of the frequency of AMPAP-mediated mEPSCs in Eps8 KO neurons upon cLTP (Menna et al 2013), which was not discussed. This reduction suggests that cells lacking Eps8 undergo depression upon cLTP treatment or that Eps8 mutant cells are not healthy. In summary, both our studies and Menna et al. showed that Eps8 is required for activity-mediated synapse formation. However, further experiments are required to demonstrate the requirement of Eps8 in activity-mediated synapse strengthening.

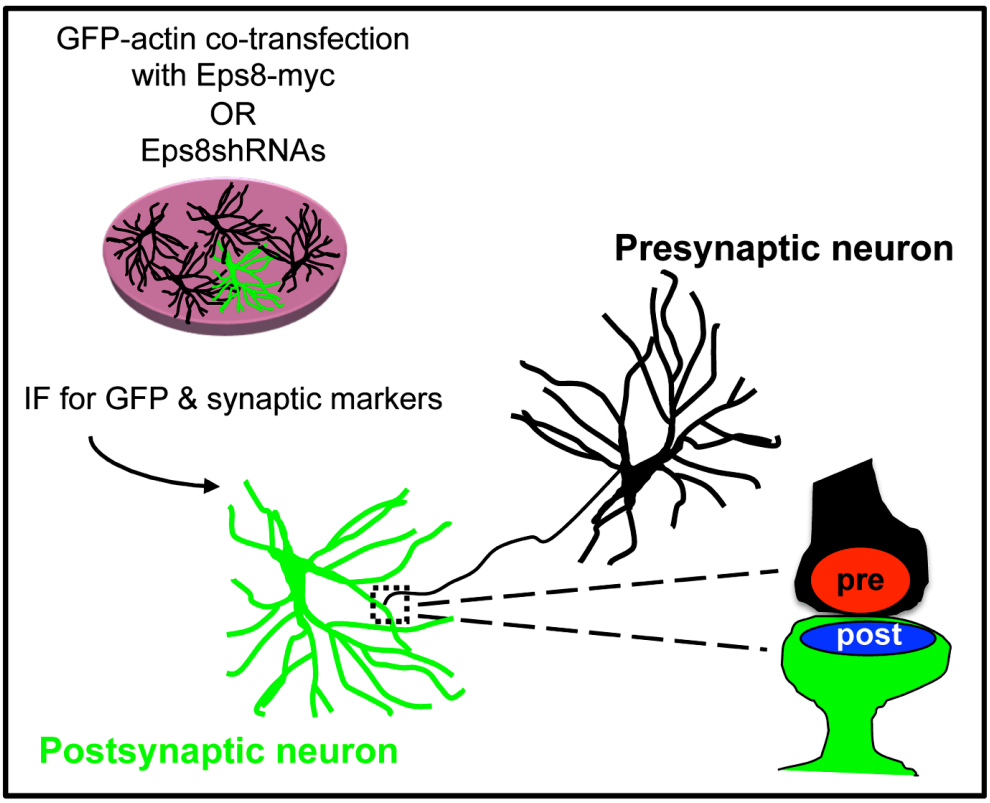



**Figure 7.6: Experimental design to study the postsynaptic role of Eps8.** Hippocampal neurons were transfected with GFP-actin to label dendritic spines and Eps8-myc or specific shRNAs against Eps8 to examine the gain and the loss of Eps8 function, respectively. The transfection protocol was standardize to have a sparse transfection. Using this approach, a GFP-positive neuron receives input almost exclusively from untransfected cells, having normal levels of Eps8. Subsequent analysis on the dendrites of the transfected cells can give information about the postsynaptic role of Eps8.

## 7.6 Conclusion & perspectives

The findings presented in this thesis reveal novel roles for the actin-binding protein Eps8 in the regulation of pre- and postsynaptic assembly and its role in the shaping of neuronal circuits by neuronal activity. We show that Eps8, which was identified as a direct interactor of the scaffold protein Dvl1, mediates Wnt-mediated axonal remodelling. In contrast, on the postsynaptic side Eps8, which is enriched in spine heads, does not mimic Wnt-mediated dendritic spine growth but induces spine formation through its actin-capping activity. Crucially, Eps8 is required for activity-induced structural and functional plasticity. Our findings provide new insights into the role of actin-bindings proteins in the remodelling of axonal terminals, the formation of dendritic spines and in activity-mediated synaptic plasticity.

Alterations in spine density and morphology has been observed in several neurological disorders characterized by cognitive impairment, such as Autism, Fragile-X syndrome, Schizophrenia and Alzheimer's disease (Penzes et al 2011a, van Spronsen & Hoogenraad 2010). In particular, Autism and Fragile-X syndrome have been associated with an abnormally high number of immature spines (Hutsler & Zhang 2010, Irwin et al 2001, Kaufmann & Moser 2000), as observed in mice lacking Eps8 (Menna et al 2013). Interestingly, Eps8 levels are decreased in the brains of individuals with autism (Menna et al 2013). These findings support the notion that defects in Eps8 function could lead to autism-like behaviours and raise the exciting possibility that manipulation of Eps8 activity could be used as a method to study and understand autism spectrum disorders.

# Appendix 1

# Wnt7a increases the phosphorylation of Cofilin

# INTRODUCTION

Actin cytoskeletal reorganization is critical for spine morphogenesis and synapse plasticity (Hotulainen & Hoogenraad 2010, Saneyoshi & Hayashi 2012, Svitkina et al 2010), but the molecular pathways that drive actin dynamics within dendritic spines remain poorly understood. One of the most well-studied actin-binding protein important for spine formation and plasticity is the actin-severing factor Cofilin (Saneyoshi & Hayashi 2012). Cofilin activity is regulated by phosphorylation/ inactivation on Serine 3 by LIMK1 and dephosphorylation/activation by the phosphatase Slingshot1 (Bernstein & Bamburg 2010). Importantly, Cofilin inactivation by LIMK1 has been implicated in spine growth and LTP (Rex et al 2009), whereas its activation has been linked to spine shrinkage and LTD (Zhou et al 2004). However, the signalling pathways that regulate Cofilin activity and its ability to modulate the actin cytoskeleton within dendritic spines are not well characterised.

Several secreted molecules that regulate spine morphogenesis and plasticity, including BDNF, EphB and Adenosine, act through the small GTPases Rac and RhoA to induce LIMK1 activation, resulting in phosphorylation/inhibition of Cofilin (Rex et al 2009, Rex et al 2007, Shi et al 2009). Previous studies from our lab have revealed that Wnt7a signalling induces spine growth through local CaMKII signalling (Ciani et al 2011). However, no link has been found between Wnt7a signalling and the actin cytoskeleton. Here we show that acute exposure to Wnt7a rapidly increases the levels of Cofilin phosphorylation on Ser3, thus inhibiting its activity, in hippocampal cultures . This effect is blocked by specific inhibitors against Rac1 and ROCK. Our results demonstrate that Cofilin is a target of Wnt signalling in neurons and provide a potential mechanism for Wnt7a-mediated spine growth.

# RESULTS

## Wnt7a inhibits Cofilin in hippocampal neurons.

To investigate whether Cofilin activity is regulated by Wnt signalling, we examined whether Wnt7a affects Cofilin phosphorylation on Ser3 in cultured hippocampal neurons. Indeed, short exposure to Wnt7a increases the ratio of

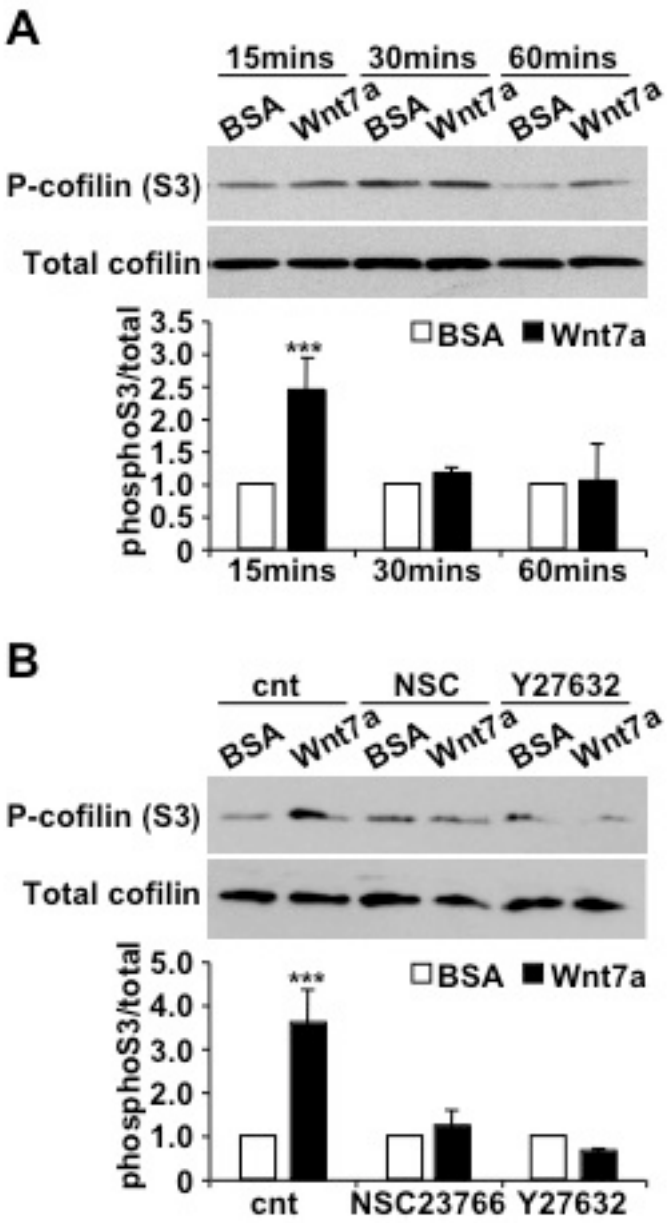



**Figure 1: Wnt7a regulates Cofilin phosphorylation on Ser3.** DIV14 hippocampal neurons were treated with Wnt7a for the indicated times (A) or for 30 mins with specific Rac1 ord ROCK inhibitors and then for 15 mins with Wnt7a (B).

phospho-Ser3/total Cofilin (Figure 1A). Cofilin phosphorylation on Ser3 is mediated by LIMK1, which is downstream of Rac1 and ROCK small GTPases. To test whether Wnt7a induces Cofilin phosphorylation through Rac1 and/or ROCK we applied Wnt7a in the presence of selective inhibitors for Rac1 (NSC23766) and ROCK (Y27632). We found that Wnt7a-mediated Cofilin phosphorylation is completely abolished in the presence of these inhibitors (Figure 1B). Together,

our results demonstrate that Wnt7a induces a rapid Cofilin inhibition through both Rac1 and ROCK small GTPases.

# DISCUSSION

Dendritic spine formation and growth is highly-dependent on remodelling of the underlined actin cytoskeleton (Bosch & Hayashi 2011, Hotulainen & Hoogenraad 2010, Lin & Webb 2009). We have previously demonstrated that postsynaptic Wnt7a signalling induces spine growth through local activation of CaMKII (Ciani et al 2011). However, the molecular mechanisms that lead to actin cytoskeletal changes during Wnt7a-mediated spine growth remain elusive.

Here I demonstrate that the actin-severing factor Cofilin is rapidly inhibited by Wnt7a through both Rac1 and ROCK small GTPases. Interestingly, upstream of both Rac1 and ROCK is CaMKII, which is required for Wnt7a-mediated increase in spine size (Ciani et al 2011). More experiments are required to examine whether inhibition of CaMKII blocks Cofilin phosphorylation on Ser3 mediated by Wnt7a. In addition, it would be also important to test whether a phospho-mutant cofilin (S3A) blocks Wnt7a-mediated spine growth.

In summary, we showed that Wnt7a regulates Cofilin function in hippocampal neurons. Our findings raise the exciting possibility that the effect of Wnt7a in spine growth is mediated by Cofilin inhibition. However, more experiments are required to support this hypothesis.

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